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Epstein-Barr virus infection in ulcerative colitis: a clinicopathologic study from a Chinese area

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Abstract

Background: Opportunistic Epstein–Barr virus (EBV) infection in patients with ulcerative colitis (UC) has attracted increasing attention. This study aimed to evaluate the clinicopathological characteristics and clinical outcomes of UC with intestinal EBV infection and to explore the predictive value of blood EBV DNA for the presence of EBV in the intestine.

Methods: Both peripheral blood and intestinal biopsies from 92 consecutive UC inpatients were included in this study. Normal colonic mucosal tissues from 20 colon cancer patients were used as controls. EBV testing and assessment were performed by EBV-DNA polymerase chain reaction (PCR), EBV-encoded small RNA *in situ* hybridization (EBER-ISH) and immunohistochemistry.

Results: A total of 36 patients (39.1%) had UC with superimposed EBV colitis [EBER greater than 2/high-power field (HPF)]. EBER counts and disease activity were significantly correlated ($p < 0.05$). The major endoscopic findings revealed more irregular and longitudinal ulcers in patients with superimposed EBV colitis ($p = 0.016$, $p = 0.021$, respectively). Age, steroid dependence, and irregular ulcerations were identified as possible risk factors. The best EBER cut-off point for outcome prediction was 2.5/HPF. At a cut-off value of 2035 copies/ml, the sensitivity and specificity of the blood EBV-DNA PCR analysis for predicting EBV presence in the intestine were 76.5% and 68.5%, respectively. EBV-infected cells in UC with high EBV concentrations mainly included B lymphocytes by clinicopathology, and the infection might have progressed from the latent to the lytic phase of the EBV life cycle.

Conclusion: The EBER count is positively correlated with disease activity. The best cut-off point for outcome prediction is 2.5/HPF. A high EBV viremia load may effectively predict EBV presence in the colonic mucosa.

Keywords: clinicopathology, Epstein–Barr virus infection, ulcerative colitis

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Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic gastrointestinal inflammatory disease characterized by constant progression and relapse. Patients with IBD, especially UC, have a higher risk of opportunistic infections, including Epstein–Barr virus (EBV) infection, due to the use of immunosuppressants as well as the disease itself.[1] EBV belongs to the herpes virus family and more than 90% of people worldwide have ever been infected.[2] The largest study to date reported that the seroprevalence of EBV infection among adult patients with IBD was 97.4%.[3] Another prospective study showed that the prevalence of EBV seronegativity in the IBD population aged 18–25 years was similar to that described in the general population and that above the age

of 25 years, seropositivity approached 100%.[4] EBV infection involves two phases, namely lytic infection and latent infection, except under specific conditions, such as infectious mononucleosis (IM), where it usually remains latent within lymphocytes for the lifetime of the host after primary acute infection. This latency is considered an escape mechanism from recognition by cytotoxic T cells, thereby allowing the virus to coexist with the host's immune system. Cellular immunity plays an important role in controlling EBV infection and maintaining the status of latency.[5–7] However, in the immunosuppressed state, EBV can not only sustain active infection but also lead to lymphoproliferative disease and occasionally lymphoma.[8–10] Due to immunosuppressive treatment for IBD and concurrent local inflammatory stimulation or malnutrition caused by the disease,[11] EBV may drive its own replication in lymphocytes that proliferate in the intestinal mucosa without being subjected to immunosurveillance. Studies have shown that the use of azathioprine (AZA) and infliximab (IFX) in patients with IBD is associated with an increased incidence of EBV opportunistic infections. The major concern among clinicians is that an EBV opportunistic infection can develop into an EBV-associated lymphoproliferative disorder. Prior studies have reported that the application of AZA and IFX and young and old age are considered risk factors for EBV-associated lymphoproliferative disorders among IBD patients.[12,13] In addition, patients with IBD have an increased risk of lymphoma, and the hazard ratio (HR) of lymphoproliferative disorders for patients receiving thiopurines *versus* those who never receive these drugs is 5.28.[9] As mentioned above, the most frequent condition in clinical practice is secondary symptomatic infection due to reactivation of the lytic phase of the virus life cycle. Therefore, the European evidence-based consensus recommends screening for EBV before the application of immunosuppressive therapy and monitoring of EBV infection.[14]

EBV commonly infects B lymphocytes, but the prevalence and phenotype of the viral infection varies significantly between countries and regions. Chronic active EBV (CAEBV) infective enteritis, a severe form of EBV-related disease in the intestine, has been found to be prevalent in east Asian countries and is characterized by clonal expansion of virally infected T or NK lymphocytes,[15,16] whereas in Western countries, CAEBV is mostly associated with B cells.[17] However, few studies have focused on this issue in UC patients with EBV infection, especially in Asian countries.

No standard diagnostic criteria are currently available for EBV opportunistic infection or EBV colitis in UC. In addition, distinguishing between latent viral infection and active EBV disease is difficult. Several techniques have been used in attempts to diagnose EBV infection, namely serology, EBV-DNA testing by polymerase chain reaction (PCR) in peripheral blood and colon mucosa, EBV-antigen testing by immunohistochemistry (IHC), and EBV-encoded small RNA (EBER) detection by *in situ* hybridization (ISH).[18–22] As a result of the high sensitivity and specificity of EBER-ISH and its precise cellular localization, some studies have recommended EBER-ISH as the gold standard for detecting EBV infection in the intestine.[23,24] To date, few studies have reported EBER findings among UC patients, especially Chinese patients. Moreover, the role of EBV infection in UC progression has not been clearly elucidated. EBV blood testing, a noninvasive and simple screening method, has also been performed in clinical practice to screen for EBV infection but few studies clearly portray the association between the presence of EBV in the blood and in tissue biopsies.[25]

Therefore, our study aimed to (a) characterize the clinical and endoscopic features of EBV opportunistic infection in the intestine, identify risk factors and explore the predictive value of blood EBV DNA for EBV presence in tissues; and (b) demonstrate the clinicopathological characteristics of EBV infection and elucidate the phenotypes of EBV-infected cells as well as possible modes of EBV infection.

Methods

Patients

We screened for EBV infection in both the blood and colonic mucosa of all UC patients who visited the First Affiliated Hospital of Sun Yat-sen University (China) for the first time between September 2016 and January 2019 and enrolled 92 subjects in this study. The inclusion criteria were (a) a diagnosis of UC based on clinical, endoscopic, radiological, and histological characteristics; (b) EBV testing in both the peripheral blood and intestinal biopsies; and (c) a regular follow-up after discharge. The exclusion criteria

were as follows: (a) concomitant opportunistic infection with other viruses or bacteria; (b) concurrent autoimmune diseases (e.g. rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus); or (c) a history of severe immunosuppressive conditions, including malignancy and post-transplantation. Medical records included demographic features, clinical characteristics, laboratory activities, endoscopic findings, and treatment regimens, among other data. Clinical disease activity was assessed by the Mayo clinical score for UC, and endoscopic activity was determined by experienced physicians using the ulcerative colitis endoscopic index of severity (UCEIS).[26,27] The Montreal classification was used to classify the behavior and location of the disease.[28] The point at which EBV was identified in a patient by more than one test and at which the results of testing were positive on one occasion was considered the start of the follow-up period; however, for patients with multiple negative results, we selected the time of the first negative biopsy as the beginning of the follow-up period. Under these circumstances, we recorded data on the clinical course according to the sample with the greatest number of positive test results. Sex- and age-matched patients who underwent colectomies for colon cancer were recruited, and their normal tumor-adjacent colonic mucosal tissues were used as controls. All participants provided written informed consent, and the study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University [Application ID: (2020) 043].

Identification of EBV infection in peripheral blood and the colonic mucosa

EBV viremia was defined as an EBV-DNA load greater than 500 copies/ml in the peripheral blood. The blood EBV-DNA load was assessed and quantified by real-time fluorescence quantitative PCR (qRT-PCR) (EBV nucleic acid fluorescence quantitative assay kit, Da An Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, China) according to the manufacturer's instructions. Because fewer than 2 ($\leq$2) EBER-positive cells per high-power field (HPF) (the highest EBV concentration per HPF) was considered to indicate nonpathogenic latent infection and might have no effect on disease progression or prognosis, we defined more than 2 EBER-positive cells/HPF ($>$2/HPF) as UC with superimposed EBV colitis in the colonic mucosa. Colonoscopy and biopsy were performed in all patients with UC. Biopsies were taken from diseased areas, usually the edges of ulcers, during colonoscopy. Normal colonic mucosal tissues adjacent to colon tumors were taken from the resected colons of colon cancer patients as controls.

EBER in situ *hybridization*

EBV infection in the intestinal mucosa was detected with the EBER ISH kit (ZSGB-BIO, Ltd., Beijing, China) following the manufacturer's instructions. *In vitro*, tissue samples were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into four-micrometer-thick slices. Before the start of ISH, slides were baked in a dry oven at 65°C for 2 hours. The prepared sections were deparaffinized with fresh xylene twice for 10 min, rehydrated with anhydrous alcohol twice for 5 min and then air-dried. Next, the dried specimens were digested by gastric enzyme for 40 min, dehydrated in a graded ethanol series and air-dried. We then incubated the slides for 3 h at 37°C with probe solution applied to each slide. Next, the slides were washed in PBS with 0.1% Tween 20 (PBST) three times for 2 min each. After application of anti-digoxin antibody labeled with horseradish peroxidase (HRP) for 30 min at 37°C, the hybridization signals were detected with a 3,3-N-diaminobenzidine tetrahydrochloride (DAB) color development system. Reliable EBER-positive staining was clearly indicated by brown-colored cell nuclei (nasopharyngeal carcinoma, which is known for its EBV positivity, was used as the positive control).

Immunohistochemistry

To determine the phenotypes of the EBV-infected cells, we performed IHC following EBER-ISH on paraffin sections. Antigen CD3 was selected as the T-cell-specific marker, while antigen CD79a was selected as the B lymphocyte-specific marker. In addition, immunohistochemical staining for BZLF1, one of the indicators of progression from the EBV latent phase to the lytic phase, was performed to classify viral behavior.[5,29,30] After deparaffinization and rehydration were carried out as previously described, antigen retrieval was performed in a pressure cooker for 3 min in 10 mM citrate buffer (0.05% Tween, pH 6.0). Then, we applied 3% hydrogen peroxidase solution for 10 min to block endogenous peroxidase activity and 1% bovine serum albumin blocking

solution for 1 h at room temperature to prevent nonspecific labeling. Slides were incubated with the primary antibody at 4°C overnight. The primary antibodies used were as follows: anti-CD3 (ZA0503, ZSGB-BIO, Ltd.), anti-CD79a (ZA0293, ZSGB-BIO, Ltd.), and anti-BZLF1 (SC-53904, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:100). Following three washes in PBST, sections were incubated with secondary antibody (#8114, #8125, Cell Signaling Technology, Inc., USA) for 30 min at room temperature. A DAB substrate kit (#8059, Cell Signaling Technology, Inc.) was used to detect the reaction reagents. For double staining of sections, visualization was performed with the ImmPress Duet Double Staining HRP/AP Polymer Kit (MP7714, Vector Laboratories Inc., USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS version 23.0 software (IBM, Armonk, NY, USA). Continuous data were described as the means ± standard deviations or the medians (lower quantiles, upper quantiles) depending on the variable distribution and were compared by *t*-test or Mann–Whitney *U* test. Categorical variables were expressed by counts and rates, and chi-square test and Fisher's exact test were used for comparisons between groups. Spearman rank correlations were calculated to assess associations between variables. To investigate the diagnostic value of peripheral blood EBV DNA by qPCR, a receiver operating characteristic (ROC) curve was drawn. The Youden index was calculated to determine the EBER cut-off value to predict outcomes. Binary multivariate logistic regression was performed to identify possible risk factors for active EBV infection in the intestine. $p < 0.05$ was considered statistically significant.

Results

Our study included 92 UC inpatients who underwent EBV testing of both peripheral blood and the intestinal mucosa at the hospital from 2016 to 2019. A total of 160 blood samples and 167 colon tissue samples were obtained because some patients had visited the hospital more than once and underwent repeated EBV testing during their follow-up visits at the hospital. The number of time-matched blood and colon tissues for the same course of disease was 128. In total, 20 normal colonic specimens from patients with colon cancer were used as controls.

EBV infection assessment in the intestinal tissues of UC patients

EBV prevalence in the intestine and clinical data. Among the 92 UC patients in the study, 36 patients (39.1%) were found to have superimposed EBV colitis (EBER > 2/HPF) (*versus* 0% for controls). The demographic features and disease characteristics are shown in Table 1. A total of 25 patients (69.4%) with superimposed EBV colitis were male, with a median age of 51.5 years, and 27 male patients (48.2%) with a younger median age of 43 years were included in the other group. The EBV colitis group included 2 (5.6%), 10 (27.8%), and 24 (66.7%) patients with mild, moderate, and severe disease, respectively, according to the Mayo clinical score. A higher percentage of severe UC and a higher median Mayo clinical score were observed for patients with superimposed EBV colitis. We also verified that the UCEIS score reflecting endoscopic disease activity was higher for patients with EBER > 2/HPF. In addition, the number of EBER-positive cells/HPF was positively correlated with the Mayo clinical score ($\rho = 0.274$, $p = 0.008$) and UCEIS score ($\rho = 0.218$, $p = 0.037$), although the correlations were weak (Figure 1). Patients who were dependent on steroids were more common in the EBV colitis group (38.9% *versus* 19.6%, $p = 0.043$), which played an important role in medication. No statistically significant differences in disease duration, disease extent or therapy-mediated alleviation of symptoms were identified between the two groups.

The clinical data of the UC patients are summarized in Table 2. Gastrointestinal symptoms in UC patients mainly included fever, abdominal pain, hematochezia, and weight loss. Fever was more often observed in patients with superimposed EBV colitis (33.3%) than in patients with EBV latent infection or no infection (14.3%) ($p = 0.031$). No differences in white blood cells, hemoglobin, blood platelets, or the erythrocyte sedimentation rate were observed between the two groups with or without EBV opportunistic infection. However, the median values for serum albumin (ALB, g/l) and C-reactive protein (CRP, mg/l) were lower and higher, respectively, among patients with superimposed EBV colitis.

Table 1. Baseline demographic and disease characteristics of UC patients.

	UC with superimposed EBV colitis (*n* = 36)	UC without EBV colitis (*n* = 56)	*p* Value
Median Age, years, (IQR)	51.5 (37.3, 60.0)	43.0 (33.3, 53.0)	**0.020**
Median disease duration, months, (IQR)	24.0 (3.8, 55.8)	29.0 (12.0, 69.0)	0.179
Male, *n* (%)	25 (69.4%)	27 (48.2%)	**0.045**
Disease extent, *n* (%)			0.684
E1 (proctitis)	0	3 (5.4%)	
E2 (left-sided colitis)	12 (33.3%)	18 (32.1%)	
E3 (extensive colitis)	24 (66.7%)	35 (62.5%)	
Clinical disease severity, *n* (%)			**0.016**
Mild or remission	2 (5.6%)	11 (19.6%)	
Moderate	10 (27.8%)	24 (42.9%)	
Severe	24 (66.7%)	21 (37.5%)	
Median Mayo clinical score, (IQR)	10 (9, 12)	9 (7, 11)	**0.018**
Endoscopic disease severity, *n* (%)			0.097
Mild or remission	16 (44.4%)	28 (50.0%)	
Moderate	12 (33.3%)	24 (42.9%)	
Severe	8 (22.2%)	4 (7.1%)	
Median UCEIS Score, (IQR)	5 (4, 6)	4.5 (3, 5)	**0.044**
Disease pattern, *n* (%)			0.017
Initial	11 (30.6%)	6 (10.7%)	
Relapse	25 (69.4%)	50 (89.3%)	
Steroid dependent, *n* (%)	14 (38.9%)	11 (19.6%)	**0.043**
Steroid resistant, *n* (%)	2 (5.6%)	2 (3.6%)	0.643
Mediations[1], *n* (%)			
5-aminosalicylates	32 (88.9%)	52 (96.3%)	0.213
Corticosteroids	22 (61.1%)	33 (61.1%)	1.000
Azathioprine	8 (22.2%)	11 (20.4%)	0.833
Methotrexate	3 (8.3%)	4 (7.4%)	1.000
Thalidomide	3 (8.3%)	1 (1.9%)	0.298
Infliximab	3 (8.3%)	2 (3.7%)	0.385
Vedolizumab	2 (5.6%)	1 (1.9%)	0.561
Adalimumab	1 (2.8%)	0	0.400

EBV, Epstein–Barr virus; Bold values, statistically significant; IQR, interquartile range; UC, ulcerative colitis; UCEIS, ulcerative colitis endoscopic index of severity. [1]Two cases were excluded from the group with UC without EBV colitis because of unknown medication history.

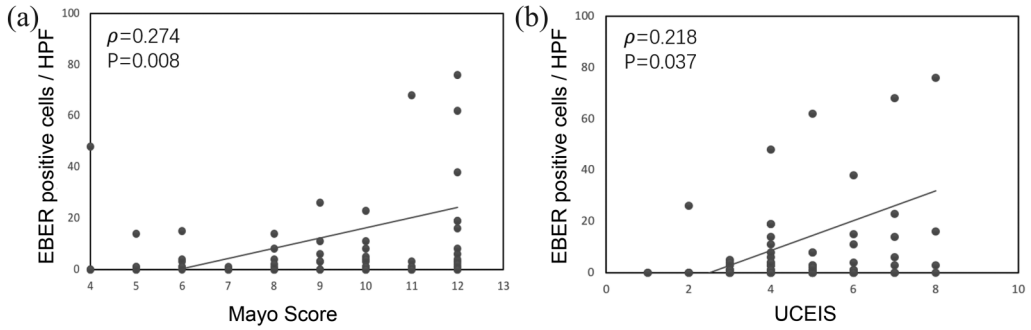

Figure 1. Correlations between EBER expression and disease activity. Spearman correlation analysis for the relationships between (a) EBER-positive cells/HPF and clinical Mayo score and (b) EBER-positive cells/HPF and the UCEIS.
EBER, Epstein–Barr virus-encoded small RNA; HPF, high-power field; UCEIS, ulcerative colitis endoscopic index.

Table 2. Clinical data of UC patients.

	UC with superimposed EBV colitis ($n=36$)	UC without EBV colitis ($n=56$)	p value
Fever, *n* (%)	12 (33.3%)	8 (14.3%)	**0.031**
Abdominal pain, *n* (%)	21 (58.3%)	36 (64.3%)	0.566
Weight loss, *n* (%)	24 (66.7%)	36 (64.3%)	0.815
Median weight loss, kg, (IQR)	3.5 (0, 8.0)	3.0 (0, 5.0)	0.226
WBC, $\times 10^{9/L}$, (IQR)	8.06 (5.12, 10.05)	8.31 (5.81, 10.28)	0.557
Hb, g/l, (IQR)	110.00 (95.25, 127.00)	119.50 (100.00, 133.00)	0.195
PLT, $\times 10^{9/L}$, (IQR)	298.50 (219.25, 408.00)	303.00 (224.25, 368.00)	0.804
ALB, g/l, (IQR)	31.50 (25.73, 34.18)	36.20 (32.00, 38.10)	**<0.001**
ESR, mm/h, (IQR)	32.50 (11.25, 48.00)	21.50 (10.50, 41.25)	0.370
CRP, mg/l, (IQR)	44.76 (13.32, 54.72)	23.70 (3.03, 29.55)	**0.011**

ALB, albumin; Bold values, statistically significant; CRP, C-reactive protein; EBV, Epstein–Barr virus; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; IQR, interquartile range; PLT, blood platelet; UC, ulcerative colitis; WBC, white blood cell.

Endoscopic findings. UC with EBV infection in the intestine manifested in various forms between patients, including diffuse and continuous inflammation, shallow ulcerations, deep and large ulcerations, and irregular and longitudinal ulcerations (Figure 2). A detailed description is provided in Table 3. Regarding endoscopic characteristics, most changes in the colonic mucosa were redness of the mucosal surface and a diffuse distribution of bleeding petechiae accompanied by edema and erosion, which were similar to the appearances of UC without any infections. Ulcers were usually found in the patients with severe disease, and the major distortions were shallow ulcerations. Moreover, we also observed that patients with superimposed colitis had more irregular and longitudinal ulcerations (33.3% and 11.1%, respectively) under endoscopy and that the differences were statistically significant. No punched-out ulcerations or cobble-like appearances were discovered. Therefore, we suggest that clinicians should be more alert and consider performing EBV testing when certain endoscopic features such as irregular and longitudinal ulcers are observed.

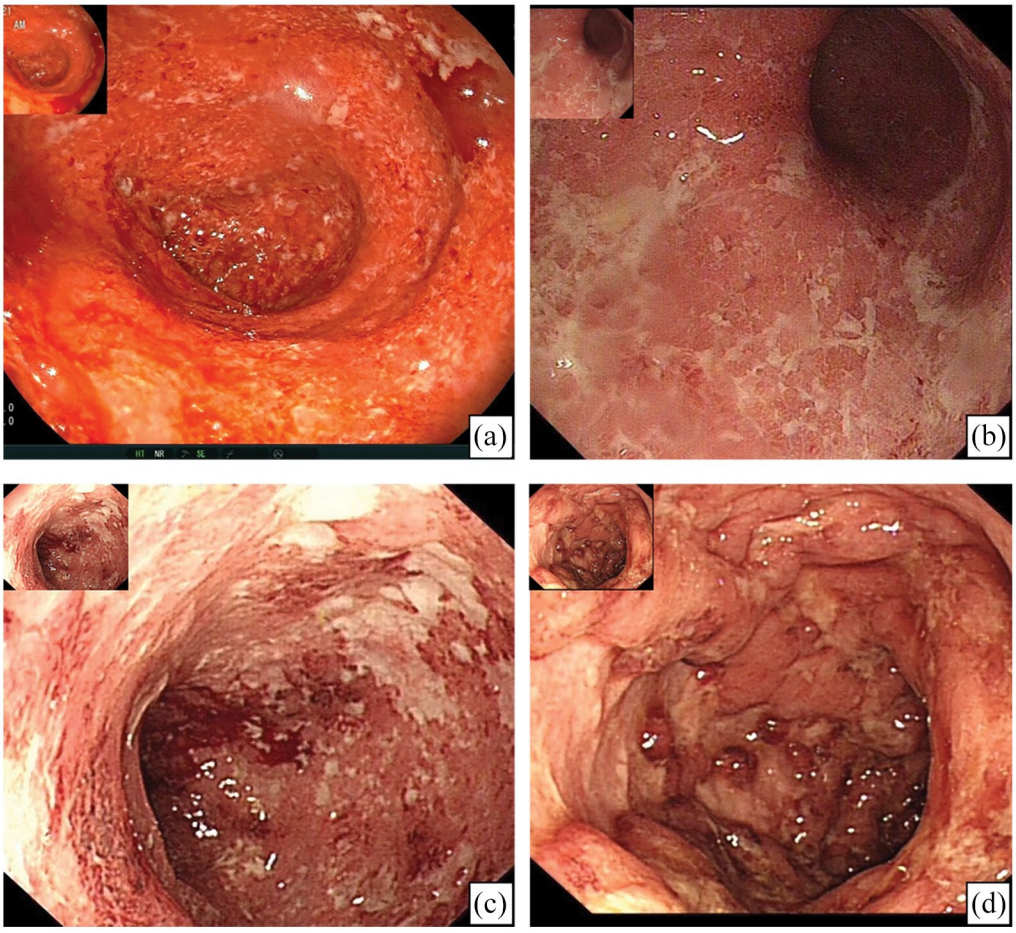

Figure 2. Endoscopic findings in the colonic mucosa of ulcerative colitis (UC) patients with Epstein–Barr virus infection. (a) Classic UC endoscopic imaging; (b) shallow ulceration; (c) irregular ulceration; (d) longitudinal ulceration.

Histopathological characteristics. Histological activity in the biopsy obtained from each patient was scored according to the Nancy histological index for UC.[31] Each case was reviewed by two experienced gastrointestinal pathologists. A five-grade classification of histological disease activity for UC was adopted (see Supplemental Figure 1). There were no cases of UC with superimposed EBV colitis among grade 0 and grade 1 cases, 23.5% among grade 2, 24.4% among grade 3 and 52.4% among grade 4 ($p=0.086$).

Histopathological features were compared between UC with superimposed EBV colitis and UC without EBV colitis. No statistically significant differences in chronic inflammatory infiltration, acute inflammatory infiltration, ulceration, mucin depletion, basal plasmacytosis, crypt architectural abnormalities, Paneth cell metaplasia or serrated architectural abnormalities were found between the two groups (Table 4).

Table 3. Endoscopic characteristics of UC patients.

	UC with superimposed EBV colitis ($n=36$)	UC without EBV colitis ($n=56$)	*p* value
Ulcerations, *n* (%)	26 (72.2%)	36 (64.3%)	0.428
Shallow ulcerations, *n* (%)	11 (30.6%)	18 (32.1%)	0.873
Longitudinal ulcerations, *n* (%)	4 (11.1%)	0 (0.0%)	**0.021**
Irregular ulcerations, *n* (%)	12 (33.3%)	7 (12.5%)	**0.016**
Lumen stenosis, *n* (%)	4 (11.1%)	3 (5.4%)	0.426

Bold values, statistically significant; EBV, Epstein–Barr virus; UC, ulcerative colitis.

Table 4. Grades of different histopathologic features between UC with superimposed EBV colitis and UC without EBV colitis.

Item	Grade	Groups		*p*
		Superimposed EBV colitis (EBER > 2/HPF)	Non-EBV colitis (EBER ⩽ 2/HPF)	
Chronic inflammatory infiltration	1	6	28	0.057
	2	15	28	
	3	5	4	
Neutrophils in the epithelium	0+1	9	34	0.168
	2	12	19	
	3	5	7	
Acute inflammatory cells infiltration	0+1	6	16	0.211
	2	13	37	
	3	7	7	
Ulceration	0	1	4	1.000
	1	25	56	
Mucin depletion	0+1	9	30	0.133
	2	11	25	
	3	6	5	
Neutrophils in lamina propria	0+1	6	12	0.256
	2	12	38	
	3	8	10	
Basal plasmacytosis	0	6	18	0.810
	1	13	28	
	2+3	5	10	
Crypt architectural abnormalities	1	6	19	0.467
	2	14	33	
	3	6	8	
Paneth cell metaplasia	0	24	53	0.856
	1	2	7	
Serrated architectural abnormalities	0+1	14	41	0.290
	2	10	17	
	3	2	2	

EBER, EBV-encoded small RNA; EBV, Epstein–Barr virus; HPF, high-power field; UC, ulcerative colitis.

Treatment and prognosis. Among the patients involved in the follow-up period, six underwent colectomy with a median EBER count of 39/HPF, all of whom were from the group with UC with superimposed EBV colitis. A statistically significant difference in the EBER count was observed between patients with and patients without surgery ($p < 0.001$), suggesting that high EBV concentrations may contribute to adverse outcomes.

A total of 20 patients (55.6%) in the group with UC with superimposed EBV colitis underwent antiviral therapy, while only 15 patients (26.8%) in the group without EBV colitis were clinically prescribed medication ($p = 0.006$). Significant differences in the frequencies and durations of hospitalization were also identified between the two groups ($p < 0.001$, $p = 0.002$, respectively). UC patients with EBV colitis may have more hospital admissions and longer hospital stays.

Risk factors. Binary multivariate logistic regression was used to assess possible risk factors between patients with and patients without UC with superimposed EBV colitis according to selected clinical and endoscopic features. We finally determined that age over 40 years [odds ratio (OR): 3.808, 95% confidence interval (CI): 1.152–12.588, $p = 0.028$], steroid dependence (OR: 6.824, 95% CI: 1.776–26.213, $p = 0.005$), and irregular ulcerations under endoscopy (OR: 4.849, 95% CI: 1.421–16.553, $p = 0.012$) were risk factors for UC with superimposed EBV colitis.

EBER cut-off point for outcome prediction. We endeavored to determine the EBER counts to predict outcomes based on a subjective assessment of patients with surgery or steroid dependence. The area under the curve (AUC) was 0.675. The best cut-off point was 2.5/HPF, with a sensitivity and specificity of 56.7% and 72.2%, respectively, indicating that when the number of inclusions is greater than 2.5/HPF, patients may be refractory.

Correlation analysis between blood and intestinal tissues

In total, 128 blood samples and colon tissues were collected simultaneously in this study. The median EBV-DNA load was 2630 copies/ml among patients with UC with superimposed EBV colitis (EBER > 2/HPF) and 658.5 copies/ml among patients without EBV colitis ($p = 0.010$). Next, we explored the accuracy of the blood assay for predicting EBV presence in tissues. The AUC for EBV-DNA levels was 0.702 (95% CI: 0.581–0.823, $p = 0.007$). When the cut-off value for blood EBV DNA was set to 2035 copies/ml, Youden's index, the sensitivity, and the specificity were 0.45, 76.5% and 68.5%, respectively.

Assessment of the phenotypes of infected cells and the phase of infection

The phenotypes of EBV-infected cells were investigated by analyzing the expression of lymphocyte-specific antigen CD3 or CD79a by IHC following EBER-ISH. All cases showed lymphoid infiltration in the intestinal mucosa, including both B lymphocytes and T lymphocytes. Antigen CD79a was detected at an average rate of 55% in EBER-positive cells from 14 of 15 selected cases; however, none of the cases showed the coexistence of EBER and CD3, suggesting that EBER-positive cells were B cells rather than T cells (Figure 3a, b).

BZLF1, an immediate-early lytic gene that initiates the EBV lytic phase of infection, was selected to determine the viral phase in patients with EBV colitis. The presence of ZEBRA protein (encoded by BZLF1) was found in 4 of 24 UC patients with high EBV concentrations (Figure 3c). Immunostaining showed that BZLF1 expression was primarily localized to lymphocytes. The four patients with ZEBRA protein had severe disease with irregular deep ulcerations, two (50%) of whom underwent colectomy due to medical treatment failure. Three of the samples (75%) had more than 50 EBER-positive cells/HPF and the fourth had 8 EBER-positive cells/HPF.

Discussion

This is the largest study to detect EBV opportunistic infection in the intestinal mucosal tissues of UC patients by EBER-ISH. We first utilized UC with superimposed EBV colitis to characterize and compare the clinical, endoscopic, and clinicopathological features of patients with and without EBV infection in the intestine. We endeavored to determine the best cut-off point of EBER for outcome prediction. Our study also indicated that a high EBV load can be more useful for predicting EBV-superimposed colitis.

The status of EBV opportunistic infection in IBD has attracted tremendous attention worldwide. A

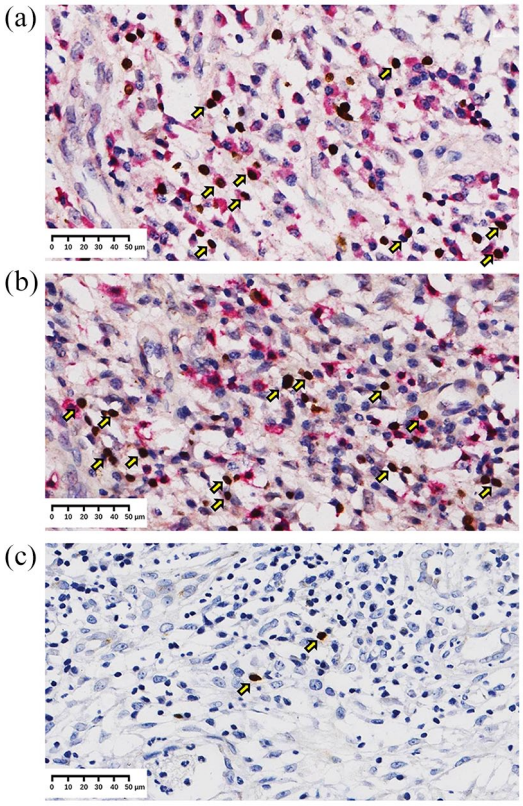

Figure 3. Phenotypes of EBV-infected cells and EBV gene expression in the infection phrase in Chinese patients with UC. (a) Antigen CD79a, a specific B-lymphocyte marker, co-localized with EBER-positive cells according to immunohistochemistry. The yellow arrows point to the positive staining (red cytoplasmic and membrane signals, brown nuclear signal); (b) No EBER-positive cells display antigen CD3 staining (T-lymphocyte marker). The yellow arrows point to EBER-positive cells (brown signal) without CD3 expression (red cytoplasmic and membrane signals); (c) A few infected lymphocytes were detected with the expression of the immediate-early lytic gene BZLF1 (brown signal), suggesting that EBV infection transitions to the lytic phase from the latent stage in some cases of UC.
EBER, EBV-encoded small RNA; EBV, Epstein–Barr virus; UC, ulcerative colitis.

considerable number of studies have demonstrated that patients with IBD, especially refractory IBD, have a higher prevalence of the virus in the intestine.[6,11,20,25,32,33] However, the results are not entirely consistent due to the patients enrolled, the study design, and, more importantly, the detection methods. In terms of diagnostic methods, various techniques can be used to detect intestinal EBV infection, such as PCR, IHC, and ISH. With the advantages of providing precise cellular localization and high sensitivity, EBER-ISH is regarded as the standard diagnostic method for EBV intestinal infection in several studies.[14,23,24] This is the first report of comprehensive EBER findings in a large cohort of UC patients in the Chinese population. Moreover, we considered that the level of EBER expression together with clinical symptoms might contribute to the problem of differentiating between EBV latent infection and colitis to some extent. According to our preliminary data and clinical experience, >2 EBER-positive cells/HPF may predict refractory UC, and a number ≤ 2/HPF can predict nonpathogenic latent EBV infection. As a result, we defined UC with superimposed EBV colitis and found that its prevalence was 39.1%. Previous studies confused viral infection with viral colitis and measured only EBER positivity, with prevalent results ranging from 41% to 60% in IBD.[6,11,20,32] As virus reactivation may worsen a patient's clinical condition and lead to refractoriness, a statistical model was developed to determine the EBER cut-off value for predicting outcomes. The model revealed that patients with more than 2.5 inclusion bodies in biopsies tended to be refractory according to statistical analysis. However, the results were not ideal for clinical practice, with 56.7% sensitivity, 72.2% specificity, and an AUC of 0.675, possibly due to the small sample size of our study. Moreover, sampling error was a potential caveat. The ability of the EBER-ISH technique to detect viral infection may add a sampling bias in the detection rate with possible underestimation of the infection, mainly because of the thinness of the slides. By contrast, previous studies have shown that PCR can be a highly sensitive and rapid method for the detection of EBV DNA and have highlighted the need to measure the mucosal viral load by PCR. Ciccocioppo[33] and colleagues discovered that all refractory patients carried mucosal viral loads greater than 10^3 copies/10^5 cells in their colonic specimens, reflecting a potential cut-off value to distinguish between EBV infection and superimposed EBV colitis. The results were significant but different from our findings when using EBER-ISH for detection. Li *et al.*[34] also reported only slight agreement between ISH and PCR. As a result, more research is needed to identify the correct diagnostic method to provide an appropriate definition of EBV colitis.

In the current study, another important finding was the positive correlation between the EBV

concentration and disease activity. A higher proportion of patients with severe disease was observed in the group with UC with superimposed EBV colitis, which is consistent with prior studies. Pezhouh *et al.* [11] discovered that EBER positivity had positive associations with the depth of inflammation and mucosal ulceration in patients with refractory IBD. Moreover, higher levels of CRP, which may indicate more active disease, were also shown among patients with EBV colitis. In addition, lower levels of ALB in UC with superimposed colitis suggested a poorer nutritional status caused by active disease. In this regard, as UC patients are immunocompromised hosts to some degree, disease activity can aggravate the immunocompromise since active disease can lead to a decline in nutritional status, and patients with malnutrition are also considered immunosuppressed.[35] These findings provide further insight into the potential role of the virus in disease progression; that is, patients with severe disease tend to have an increased incidence of EBV reactivation, and the virus might subsequently worsen the situation. Indeed, viral replication can be a consequence of the local immunosuppression induced by high disease activity and local inflammatory stimulation caused by intestinal barrier dysfunction with or without the impact of medications. Our study showed a higher proportion of steroid-dependent patients among those with superimposed EBV colitis. However, no significant differences in therapy-mediated alleviation of symptoms were noted, which is consistent with other studies.[11]

EBV-DNA detection in the blood by qPCR and serum EBV-specific antibodies are also major means of detecting EBV infection in EBV-associated diseases. Blood testing is noninvasive, objective, and simple. An increased number of EBV-DNA copies can be detected in the peripheral blood, especially during the symptomatic phases of some diseases.[30] Magro *et al.*[12] declared that IBD was a risk factor for the presence of EBV DNA in the blood. In the present study, a DNA blood test revealed a positive correlation with intestinal tissue ISH for EBER. In addition, the results of blood DNA testing were compared with assess this method of detection in colon tissues previously confirmed to exhibit EBV opportunistic infection by EBER-ISH. The blood test showed 76.5% sensitivity and 68.5% specificity when the cut-off value for EBV DNA was set to 2035 copies/ml. Although the sensitivity and specificity were not sufficiently high, a higher EBV copy number might predict the presence of EBV in the colon more accurately than the traditional 500 copies/mL to some extent. However, different findings have been reported for the association between the presence of EBV in the blood and in biopsies. A prior study involving only 27 patients reported no correlation between the presence of EBV in the blood and in biopsies.[25] We attributed this contradiction to the sample sizes of the studies because the available number of subjects in their study was too small. When considering serological testing to assess viral infection, positivity of the IgG antibody suggests prior exposure, while IgM positivity implies recent infection, possibly indicating virus reactivation in adult IBD.[14] If viral reactivation is associated with relapse of the underlying IBD with or without treatment, the prevalence of serum anti-virus IgM antibodies will increase. However, a lack of IgM positivity may be a false negative in IBD patients under immunosuppressive and/or immunomodulatory treatment. A further limitation in measuring serum antibodies is the fact that elevated levels of IgM can persist for up to two years after infection, and immunocompromised patients may not mount an IgM response.[33] Our findings partly support this view as no cases were identified as IgM-positive in this cohort of UC patients (data not shown). Taken together, the role of blood testing may be limited and detection of EBV in the blood may not correlate with viral colitis, indicating that IBD with opportunistic infection in the colon might exist independently of systemic involvement.

At present, determining when EBV plays a vital role in mucosal inflammation and disease refractoriness as EBV colitis or when EBV is only a nonpathogenic bystander is difficult. BZLF1, an immediate-early lytic gene that initiates the switch from the latent phase to the lytic phase of EBV infection, was selected to determine whether the virus was reactivated in UC.[36] Patients with BZLF1-immunostained tissues in this study were more likely to have more severe disease activity with irregular deep ulcers, suggesting that EBV reactivation might lead to a worse outcome. However, some questions still remain. First, in 16.7% of the patients, BZLF1 immunostaining alone was not sufficient to determine whether the patients with EBV colitis had a lytic infection. As previously observed in other studies on IBD and IM,[5,6,37] the number of BZLF1-positive cells was actually small. We doubt that immunochemistry

is sufficiently sensitive to differentiate lytic from latent infection. Second, some studies have reported a relationship between epithelial cells and EBV lytic infection in epithelial and lymphoid tumors. The role of epithelial cells in the EBV life cycle is to support the replication and spread of the virus within the host.[37,38] In the current study, neither BZLF1-stained cells nor EBER-positive cells were identified in the epithelial cells of UC patients with EBV colitis. However, the lack of BZLF1 expression in epithelial cells cannot rule out the presence of productive viral replication. Although UC involves the inflamed colonic mucosa, we do not think that the intestinal epithelium is the only site of viral replication. UC with EBV colitis, as an inflammatory lesion, is preferable to the involvement of B-lymphoid cells and thus infects the colonic mucosa through mucosal immunity. Resting memory B cells differentiate into plasma cells as an inflammatory stimulus to trigger the EBV lytic phase,[36] and this process can worsen clinical symptoms and induce refractoriness.[39,40]

This is the first study to verify the clinicopathological characteristics of Asian UC patients in whom EBV-infected cells were mainly B lymphocytes. No EBV-infected T cells were identified in the present study. Our observations are consistent with prior research in Western countries[6] and suggest that the mechanism of EBV involvement in IBD with viral colitis is the same between the East and West; however, this finding differs from that for phenotypes of CAEBV and lymphoma between the East and West. EBV replication has been proven to be associated with B-cell infiltration and proliferation in IBD patients, although impaired T-cell immunity is thought to be critical in controlling viral replication.[39] In Spieker's study, no more than 30% of the EBER-positive cells stained positive for B-cell marker antigen CD20. However, in our study, a larger proportion of EBER-positive cells (55%) was found by immunostaining with the use of another B-cell antigen, namely, CD79a. The expression of CD20 in B lymphocytes was downregulated during the period of differentiation into plasma cells, while CD79a was expressed in the plasma cells. Colocalization with CD79a indirectly highlighted the previous statement that EBV reactivation might trigger alterations in plasma cells and indicate initiation of the lytic phase, providing evidence for viral reactivation in UC.

Several limitations exist in our study. First, this is a case-control study from one of the largest tertiary IBD centers in China. All data were collected from inpatients, most of whom had moderate-to-severe disease activity. We could not generate definitive diagnostic criteria for EBV colitis according to clinical disease activity or endoscopic activity. A prospective study should be performed to further characterize IBD with EBV colitis in the future. In addition, the small sample size was a shortcoming and may account for our finding of only a weak correlation between EBER expression and disease activity. Finally, our study lacks an analysis of antiviral therapy, although no efficacious antiviral therapy is available for EBV infection.

In conclusion, a combined assessment of the clinical, endoscopic, and pathological features of UC patients with EBV opportunistic infection is summarized in the current study. These significantly different features can help clinicians better assess the presence of EBV in the intestine and superimposed EBV colitis. Furthermore, the EBV clinicopathological characteristics of the EBV infection phases and phenotypes also demonstrate EBV reactivation and disease progression in some UC patients. Further study is still needed to evaluate the association between EBV infection and IBD and to clarify the potential role of the virus.

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Author contributions

Yao He and Ziyin Ye developed the concept of and designed the study, guided the project and revised the manuscript; Shu Xu collected the medical records, conducted the experiments, performed the statistical analysis and wrote the manuscript; Haiyang Chen conducted the experiments and analyzed the data; Xiaoman Zu and Xiuxue Hao collected the clinical information; Rui Feng, Shenghong Zhang, and Baili Chen assessed the clinical and endoscopic disease activity of the enrolled subjects and provided advice for the study; Zhirong Zeng and Minhu Chen supervised the

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

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References

1. Ordás I, Feagan BG and Sandborn WJ. Early use of immunosuppressives or TNF antagonists for the treatment of Crohn's disease: time for a change. *Gut* 2011; 60: 1754–1763.
2. Cohen JI. Epstein-Barr virus infection. *N Engl J Med* 2000; 343: 481–492.
3. De Francisco R, Castano-Garcia A, Martinez-Gonzalez S, *et al*. Impact of Epstein-Barr virus serological status on clinical outcomes in adult patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2018; 48: 723–730.
4. Linton MS, Kroeker K, Fedorak D, *et al*. Prevalence of Epstein-Barr Virus in a population of patients with inflammatory bowel disease: a prospective cohort study. *Aliment Pharmacol Ther* 2013; 38: 1248–1254.
5. Niedobitek G, Agathanggelou A, Herbst H, *et al*. Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells. *J Pathol* 1997; 182: 151–159.
6. Spieker T and Herbst H. Distribution and phenotype of Epstein-Barr virus-infected cells in inflammatory bowel disease. *Am J Pathol* 2000; 157: 51–57.
7. Kempkes B and Robertson ES. Epstein-Barr virus latency: current and future perspectives. *Curr Opin Virol* 2015; 14: 138–144.
8. Kandiel A, Fraser Ag, Korelitz Bi, *et al*. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. *Gut* 2005; 54: 1121–1125.
9. Beaugerie L, Brousse N, Bouvier AM, *et al*. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. *Lancet* 2009; 374: 1617–1625.
10. Siegel CA, Marden SM, Persing SM, *et al*. Risk of lymphoma associated with combination anti-tumor necrosis factor and immunomodulator therapy for the treatment of Crohn's disease: a meta-analysis. *Clin Gastroenterol Hepatol* 2009; 7: 874–881.
11. Pezhouh MK, Miller JA, Sharma R, *et al*. Refractory inflammatory bowel disease: is there a role for Epstein-Barr virus? A case-controlled study using highly sensitive Epstein-Barr virus-encoded small RNA1 in situ hybridization. *Hum Pathol* 2018; 82: 187–192.
12. Magro F, Santos-Antunes J, Albuquerque A, *et al*. Epstein-Barr virus in inflammatory bowel disease-correlation with different therapeutic regimens. *Inflamm Bowel Dis* 2013; 19: 1710–1716.
13. Hradsky O, Copova I, Zarubova K, *et al*. Seroprevalence of Epstein-Barr virus, cytomegalovirus, and polyomaviruses in children with inflammatory bowel disease. *Dig Dis Sci* 2015; 60: 3399–3407.
14. Rahier JF, Magro F, Abreu C, *et al*. Second European evidence-based consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease. *J Crohns Colitis* 2014; 8: 443–468.
15. Kimura H, Hoshino Y, Kanegane H, *et al*. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 2001; 98: 280–286.
16. Liu R, Wang M, Zhang L, *et al*. The clinicopathologic features of chronic active Epstein-Barr virus infective enteritis. *Mod Pathol* 2019; 32: 387–395.

17. Cohen JI, Jaffe ES, Dale JK, *et al.* Characterization and treatment of chronic active Epstein-Barr virus disease: a 28-year experience in the United States. *Blood* 2011; 117: 5835–5849.

18. Glickman JN, Howe JG and Steitz JA. Structural analyses of EBER1 and EBER2 ribonucleoprotein particles present in Epstein-Barr virus-infected cells. *J Virol* 1988; 62: 902–911.

19. Wakefield AJ, Fox JD, Sawyerr AM, *et al.* Detection of herpesvirus DNA in the large intestine of patients with ulcerative colitis and Crohn's disease using the nested polymerase chain reaction. *J Med Virol* 1992; 38: 183–190.

20. Yanai H, Shimizu N, Nagasaki S, *et al.* Epstein-Barr virus infection of the colon with inflammatory bowel disease. *Am J Gastroenterol* 1999; 94: 1582–1586.

21. Young LS and Murray PG. Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene* 2003; 22: 5108–5121.

22. Hayden RT, Yan X, Wick MT, *et al.* Factors contributing to variability of quantitative viral PCR results in proficiency testing samples: a multivariate analysis. *J Clin Microbiol* 2012; 50: 337–345.

23. Khan G. Screening for Epstein-Barr virus in Hodgkin's lymphoma. *Methods Mol Biol* 2009; 511: 311–322.

24. Gulley ML and Tang W. Laboratory assays for Epstein-Barr virus-related disease. *J Mol Diagn* 2008; 10: 279–292.

25. Nissen LH, Nagtegaal ID, De Jong DJ, *et al.* Epstein-Barr virus in inflammatory bowel disease: the spectrum of intestinal lymphoproliferative disorders. *J Crohns Colitis* 2015; 9: 398–403.

26. Travis SP, Schnell D, Krzeski P, *et al.* Developing an instrument to assess the endoscopic severity of ulcerative colitis: the ulcerative colitis endoscopic index of severity (UCEIS). *Gut* 2012; 61: 535–542.

27. Magro F, Gionchetti P, Eliakim R, *et al.* Third European evidence-based consensus on diagnosis and management of ulcerative colitis. Part 1: definitions, diagnosis, extra-intestinal manifestations, pregnancy, cancer surveillance, surgery, and ileo-anal pouch disorders. *J Crohns Colitis* 2017; 11: 649–670.

28. Satsangi J, Silverberg MS, Vermeire S, *et al.* The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006; 55: 749–753.

29. Ma SD, Hegde S, Young KH, *et al.* A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. *J Virol* 2011; 85: 165–177.

30. Kerr JR. Epstein-Barr virus (EBV) reactivation and therapeutic inhibitors. *J Clin Pathol* 2019; 72: 651–658.

31. Marchal-Bressenot A, Salleron J, Boulagnon-Rombi C, *et al.* Development and validation of the Nancy histological index for UC. *Gut* 2017; 66: 43–49.

32. Bertalot G, Villanacci V, Gramegna M, *et al.* Evidence of Epstein-Barr virus infection in ulcerative colitis. *Dig Liver Dis* 2001; 33: 551–558.

33. Ciccocioppo R, Racca F, Paolucci S, *et al.* Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: need for mucosal viral load measurement. *World J Gastroenterol* 2015; 21: 1915–1926.

34. Li X, Chen N, You P, *et al.* The status of Epstein-Barr virus infection in intestinal mucosa of Chinese patients with inflammatory bowel disease. *Digestion* 2019; 99: 126–132.

35. Sands BE, Cuffari C, Katz J, *et al.* Guidelines for immunizations in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2004; 10: 677–692.

36. Murata T and Tsurumi T. Switching of EBV cycles between latent and lytic states. *Rev Med Virol* 2014; 24: 142–153.

37. Ciccocioppo R, Racca F, Scudeller L, *et al.* Differential cellular localization of Epstein-Barr virus and human cytomegalovirus in the colonic mucosa of patients with active or quiescent inflammatory bowel disease. *Immunol Res* 2016; 64: 191–203.

38. Temple RM, Zhu J, Budgeon L, *et al.* Efficient replication of Epstein-Barr virus in stratified epithelium in vitro. *Proc Natl Acad Sci U S A* 2014; 111: 16544–16549.

39. Sankaran-Walters S, Ransibrahmanakul K, Grishina I, *et al.* Epstein-Barr virus replication linked to B cell proliferation in inflamed areas of colonic mucosa of patients with inflammatory bowel disease. *J Clin Virol* 2011; 50: 31–36.

40. Taoka K, Nannya Y, Yamamoto G, *et al.* Progressive transition of Epstein-Barr virus associated lymphoproliferative disease subtypes with the development of lung cancer. *Am J Hematol* 2009; 84: 600–603.