Combined Infection of Human Herpes Viruses: A Risk Factor for Subsequent Colectomy in Ulcerative Colitis

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Title

Combined infection of human herpes viruses: a risk factor for subsequent colectomy in ulcerative colitis

Short title

HHV in IBD

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Abbreviations

AZA, azathioprine; CD, Crohn's disease; CI, confidence interval; CMV, cytomegalovirus; CyA, cyclosporine A; EBV, Epstein-Barr virus; HHV, human herpes virus; HR, hazard ratio; HSV, herpes simplex virus; IBD, inflammatory bowel disease; IFX, infliximab; MES, Mayo endoscopic subscore; PCR, polymerase chain reaction; TAC, tacrolimus; TNF, tumor necrosis factor; UC, ulcerative colitis; VZV, Varicella-Zoster virus; 5-ASA, 5-aminosalicylic acid; 6-MP, 6-mercaptopurine

Abstract

Background: Little is known about the prevalence and pathogenicity of human herpes viruses except for cytomegalovirus (CMV) in patients with inflammatory bowel disease (IBD). The aim of this study was to determine the prevalence of human herpes viruses on colonic mucosa in patients with IBD and assess the long-term outcomes in these patients.

Methods: We examined the colonic mucosal specimens from 66 patients with ulcerative colitis (UC), 54 patients with Crohn's disease (CD), and 29 healthy patients to identify the six most common types of human herpes virus, using multiplex polymerase chain reaction (PCR) technique.

Results: Herpes simplex virus (HSV)-1/2 and varicella-zoster virus (VZV) were not detected in any of the groups. There was a higher prevalence of Epstein-Barr virus (EBV) (21.2%) and CMV (15.1%) in patients with UC than in patients with CD (EBV 9.3%, CMV 0%) and the healthy control group (EBV 0%, CMV 3.4%). The prevalence of human herpes virus (HHV)-6A/B and HHV-7 was not statistically different among the groups. Five UC patients with inflammation had coexisting CMV and EBV or HHV-6. The combined infection of CMV with EBV or HHV-6 was a significant and

independent prognostic factor for subsequent colectomy in patients with UC.

Conclusions: The increased prevalence of CMV coexisted with EBV/HHV-6 infection was associated with the clinical course in patients with UC.

Keywords

human herpes virus, Epstein-Barr virus, cytomegalovirus, human herpes virus-6, ulcerative colitis, Crohn's disease

Introduction

Crohn's disease (CD) and ulcerative colitis (UC), which are chronic and relapsing forms of inflammatory bowel disease (IBD), have been suggested to be complex multifactorial diseases, with genetic factors, mucosal barrier function, immune response, and environmental factors, including infectious agents playing a role ^{1,2}. Although no infectious agent has been identified as the cause of IBD, many pathogens have been suggested to be a trigger and/or refractory factor in patients with IBD ³. Recently, loss of immune tolerance to commensal enteric bacteria is hypothesized to be one of the pathogenic mechanisms of IBD. Several IBD susceptibility genes associated with innate immunity and autophagy (e.g., *NOD2/CARD15*, *ATG16L1*, *IRGM*) contribute to this phenomenon ^{4,5}. The reactivation of herpes simplex virus type (HSV)-1, varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpes virus type (HHV)-6, which belong to the herpesviridae family of viruses with a double-stranded DNA, cause several symptoms in severe immunocompromised hosts, such as individuals who undergo hematopoietic stem cell transplantation ^{6,7}. CMV reactivation was frequently identified in the colonic mucosa of IBD patients, and this was associated with a significant clinical morbidity, such as a toxic megacolon,

requirement of colectomy, and high mortality rate ^{8,9}. Several studies have described the significant presence of EBV in colonic specimens of IBD patients ^{10,11,12}. In contrast, there are few reports describing other human herpes viruses in patients with IBD. A recent study showed that 26.8% and 10.1% of UC patients are positive for EBV and HHV-6 in stool samples, respectively ¹³. This study also showed that 22.4% and 1.7% of active UC patients had combined prevalence of CMV with EBV and CMV with HHV-6, respectively, implicating that combined infection may be related to the pathogenesis of UC. More recent research evaluated human herpes viruses by PCR in colonic mucosa of immunocompetent patients, IBD patients, and human immunodeficiency virus-infected patients, which revealed that the positivity rates of EBV and CMV, but not of the other human herpes viruses, increased in the order of the immunocompetent patients, the IBD patients, and the human immunodeficiency virus-infected patients (EBV, 23.1%, 53.7%, 72.7%; CMV, 7.7%, 24.4%, 54.5%, respectively) ¹⁴. However, there are no studies demonstrating the clinical meaning of other human herpes viruses or combined infection of human herpes viruses with CMV in IBD.

The aim of this study was to determine the prevalence and the risk factors of human

herpes virus family on colonic mucosa in patients with IBD. In addition, we investigated the long-term outcomes in these patients.

Materials and Methods

Patients and Samples

The diagnosis of IBD is based on clinical, endoscopic, and histopathological findings. Mucosal specimens were collected between January 2007 and September 2010 with biopsies from the rectosigmoid colon during colonoscopy or surgical resected specimens of the rectosigmoid colon. For comparison, control biopsy specimens were taken from the normal rectosigmoid mucosa of patients who underwent colonoscopy for various reasons (e.g., cancer screening). Endoscopic activity was determined on rectosigmoid colon for CD, using an endoscopic score of 0–2 (0, normal; 1, light erythema or granularity; 2, granularity, friability, and bleeding, with or without ulcerations) according to Froslic's criteria ¹⁵, with a score of 0 or 1 regarded as noninflamed. For UC, endoscopic activity was assessed by Mayo endoscopic subscore (MES) ¹⁶, with a score of 0 regarded as noninflamed. Montreal classification ¹⁷ was used to classify the disease extent, which included proctitis (E1), left-sided colitis (E2),

or Pancolitis (E3) for UC; disease location included ileal (L1), colonic (L2), or ileo-colonic (L3); and behavior included inflammatory (B1), stricturing (B2), or penetrating disease (B3) for CD.

Multiplex polymerase chain reaction

DNA was extracted from sigmoid colon samples, using QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. To detect and identify the 6 most common types of human herpes virus (HSV-1/2, VZV, EBV, CMV, HHV-6A/B, and HHV-7), we used multiplex polymerase chain reaction (PCR) technique and specific primers as described previously¹⁸ (Table 2). Each primer pair was designed with different amplicon size to identify each product with agarose gel electrophoresis. PCR was performed in 50 μ L volumes, using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 μ M of each primer, and 5 μ L of DNA extract. After initial denaturing DNA at 95°C for 10 minutes, the amplification reaction was carried out in a Veriti 96-well thermal cycler (Applied Biosystems) with preliminary denaturation for 10 minutes at 95°C, followed by touchdown protocol for the first 10 cycles of denaturation at 95°C for 30 seconds,

annealing at 70°C to 61°C for 30 seconds with decrease of 1°C for each cycle, and extension at 72°C for 1 minute. Afterward, additional 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds were carried out. The amplified products were visualized by ethidium bromide staining and UV transillumination, following electrophoretic separation on 3% agarose gels. A combined infection group was defined as patients positive for DNA of two or more human herpes viruses, and single-infection group was defined as patients positive for DNA of only one human herpes virus.

Statistical analysis

Continuous variables are represented as median and interquartile range (IQR). Fisher's exact test was used to compare the differences in the prevalence of each viral infection among groups. Factors associated with viral infection were analyzed, using a univariate logistic regression model. Subsequently, factors with $P < 0.2$ in univariate analysis were included in the multivariate analysis. The data were presented as odds ratio (OR) with 95% confidence intervals (CI). Risk factors for colectomy were analyzed, using univariate and multivariate Cox proportional hazard model with adjustment for partial Mayo score, Mayo endoscopic subscore (MES), serum albumin

level, serum C-reactive protein level, and anti-viral therapy.. The data were presented as hazard ratios (HR) with 95% CI. Differences were considered significant when P values were less than 0.05. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More precisely, it is a modified version of R commander (version 1.6-3) that includes statistical functions frequently used in biostatistics.

Ethical Considerations

Ethics Committee of Osaka City University approved this study to obtain the mucosal specimens (protocol number 2286). Informed consent was obtained from all patients and healthy controls. The protocol for obtaining and analyzing long-term outcomes (protocol number 3712) was approved by the ethics committee of Osaka City University Hospital. The ethics committee granted exemption from written informed consent for the long-term analysis because the analysis used anonymized clinical data that were retrospectively obtained after each patient agreed to treatment by written consent. Nevertheless, all patients were notified of the content and information on this

study, and they were given the opportunity to refuse participation. None of the patients refused participation. This procedure followed the Ethical Guidelines for Medical and Health Research Involving Human Subjects established by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare in Japan.

Results

Study population and prevalence of human herpes viruses in colonic mucosa by multiplex polymerase chain reaction

Twenty-nine healthy controls, 66 patients with UC, and 54 patients with CD were included in this study. The median age of the controls, the patients with UC, and the patients with CD was 64.9 years (IQR, 58–73 years), 48.3 years (IQR, 34.5–60.8 years), and 36.9 years (IQR, 28.3–42 years), respectively. The percentage of mucosal inflammation in patients UC and CD patients was 54.5% (36 inflamed and 30 noninflamed specimens) and 25.9% (14 inflamed and 40 noninflamed specimens), respectively. Mesalamine, corticosteroids, immunomodulators (azathioprine or 6-mercaptopurine), calcineurin inhibitors (cyclosporine or tacrolimus), and infliximab

were used in 81.8%, 30.3%, 12.1%, 6.8% and 1.5% of UC patients and 75.9%, 3.7%, 35.2%, 0.0% and 37.0% of CD patients, respectively (Table 1).

HSV-1/2 and VZV were not detected in the colonic mucosa of any of the patients (Table 3). EBV was found in 14 of the 66 (21.2%) patients with UC and five of the 54 (9.3%) patients with CD; however, none of the subjects in the control group was positive for EBV. The difference in the prevalence of EBV infection among patients with UC, patients with CD, and the healthy controls was significant ($P < 0.001$ by Fisher's exact test). CMV was found in one of the 29 (3.4%) healthy controls, 10 of the 66 (15.1%) UC patients, and none of the 54 CD patients. The difference in the prevalence of CMV infection among patients with UC, patients with CD, and the healthy controls was significant ($P = 0.003$). HHV-6A/B was detected in two of the 29 (6.9%) subjects in the control group, six of the 66 patients with (9.1%) UC, and five of the 54 (9.3%) patients with CD. There was no difference in the prevalence of HHV-6 infection among patients with UC, patients with CD, and the healthy controls ($P = 1.000$). None of the 29 subjects in the control group had HHV-7, while it was found in one of the 66 (1.5%) patients with UC, and two of the 54 (3.7%) patients with CD. There was no difference in the prevalence of HHV-7 infection among patients with UC,

CD, and the healthy controls ($P = 0.594$). Analysis of relation between the prevalence of human herpes viruses and endoscopic severity according to Mayo endoscopic subscore (MES) showed that severe colitis (MES 2 or 3) of UC had higher prevalence of CMV infection, MES 0, 3.3%; MES 1, 0%; MES 2, 29.4%; MES 3, 28.6% ($P = 0.019$), but not EBV and HHV-6A/B (Table 4).

Five out of the total 149 patients had the combined infection with EBV and CMV, and all five were in patients with UC and inflammation. One out of the total 149 patients had the combined infection with EBV and HHV-6; one out of 149 patients had the combined infection with CMV and HHV-6. All the patients with combined infections were those with UC (Fig. 1). None of the patients were infected with HHV-7 and other viruses.

Associations between baseline characteristics and the human herpes virus infection in inflammatory bowel disease

Recent meta-analysis showed that use of corticosteroids and thiopurines are associated with CMV reactivation in IBD ¹⁹. To identify factors associated with EBV, CMV, and HHV-6 infection in IBD, the factors, including concomitant medications

were analyzed using logistic regression analysis. Univariate logistic regression analysis revealed that existence of mucosal inflammation and use of corticosteroids, cyclosporine A (CyA), or tacrolimus (TAC) were significant risk factors for EBV infection (Table 5). Multivariate logistic regression analysis also identified the existence of mucosal inflammation as a significant and independent risk factor for EBV infection (OR: 7.56, $P = 0.002$) (Table 6). Existence of mucosal inflammation and use of corticosteroids were identified as significant risk factors for CMV infection by univariate logistic regression analysis (Table 5). Multivariate logistic regression analysis revealed that older age (OR: 1.06, $P = 0.054$) and use of corticosteroids (OR: 5.24, $P = 0.064$) tended to increase the risk of CMV infection (Table 6). We found no significant risk factors associated with the HHV-6 infection in our univariate and multivariate analyses (Table 5 and 6).

Association between combined infection of human herpes viruses and long-term outcome of ulcerative colitis

A recent study has shown that about one-fourth of active UC patients had combined prevalence of EBV or HHV-6 with CMV ¹³. Our study also showed that 10.6% of

patients with UC had combined infection, but none of the patients with CD had a combined infection. The question here was whether the combined infection in colonic mucosa of UC patients was the primary cause of mucosal inflammation or a secondary event to severe disease. To address the question, we next evaluated the clinical course of patients with UC. The median duration of follow-up was 78.2 months (IQR, 46.3–81.5 months). Univariate COX proportional hazard analysis revealed a significant relationship between colectomy-free survival and combined infection ($P = 0.019$) (Table 7). Multivariate analysis adjusted for partial Mayo score, Mayo endoscopic subscore (MES), serum albumin level, serum C-reactive protein level, and anti-viral therapy showed that combined infection was significant and independent prognostic factors for subsequent colectomy (adjusted HR = 72.3, 95% CI: 1.85–2.82E+03, $P = 0.022$) (Table 7), although single infection was not significant and was not an independent prognostic factor ($P = 0.824$). The benefit of antiviral therapy on the long-term outcome of UC patients with CMV has not been well established^{20,21}. Six UC patients with CMV were followed to analyze long-term outcome in this study. Four CMV positive patients had mucosal inflammation, and two of them were treated by anti-viral therapy by ganciclovir. Therefore, we assessed anti-viral therapy as a risk factor for subsequent

colectomy, which showed no efficacy of the anti-viral therapy in this study (multivariate Cox proportional analysis, $P = 0.595$) (Table 7).

Discussion

In this study, we demonstrated higher prevalence of CMV and EBV in colonic mucosa of patients with UC than in healthy controls and patients with CD. We also showed that a combined infection of EBV or HHV-6 with CMV is independently and significantly associated with an increased risk of colectomy in UC.

Eight types of herpes viruses are known to be pathogenic (HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, and HHV-8) ⁶. In the present study, we used the multiplex PCR technique for the diagnosis of these human herpes infections in the colonic mucosa of 29 healthy patients and 120 patients with IBD (UC or CD). There are several methods for detecting these viral infections, such as histology (including immunohistochemistry), serologic assays, culture, PCR for viral DNA, and viral antigenemia. PCR is reported to be the most sensitive way to detect these viruses ²². The multiplex PCR technique we used is of high specificity and sensitivity; the detection limit for each virus was approximately 20 DNA copies per reaction. Moreover, this

method does not need expensive equipment and complicated processes ²³. Therefore, multiplex PCR is an economical, quick, and useful screening method for such viral infection.

None of the subjects, both the healthy controls and those with IBD, had HSV-1/2 or VZV in their colonic mucosa. The most common site of the HSV involvement in immunocompromised host is the esophagus, and the involvement of the colon is very rare ²⁴. Only a few cases of HSV colitis in IBD have been described in the English-language literature ²⁵⁻²⁷. Patients with IBD who use immunosuppressive medications are at high risk for VZV reactivation ²⁸; however, the involvement of the colon is also very rare ²⁹.

HHV-7 infection was found infrequently in our study, and the prevalence of the HHV-7 infection did not differ between the control group and the group with IBD patients. A recent study evaluating human herpes viruses showed that 30.8% of immunocompetent patients and 39.0% of IBD patients were positive for HHV-7, although the positivity rates did not differ between each group ¹⁴. The positivity rates of human herpes viruses (EBV, CMV, HHV-6, and HHV-7) in the study were higher than ours. This discrepancy might be due to different assay (standard vs multiplex PCR,

different primer pair) or different background of patients. Information on the pathogenic role of HHV-7 reactivation in immunocompromised host is still very limited ³⁰.

Nineteen (15.8%) patients with IBD were positive for EBV in the present study, which is the highest prevalence reported. The prevalence of EBV in IBD patients was significantly higher than that in the controls. The association between EBV infection and the development of neoplasia has been known, especially in immunocompromised host ³¹. Several studies have described the significant presence of EBV in colonic specimens of IBD patients compared to the control group ^{10,11,12,32}. Recently, Sankaran-Walters et al. reported that proliferating B-lymphocytes in inflamed colonic mucosa of IBD patients expressed high EBV genes ³³. Weinberg et al. reported a patient with IBD whose condition was exacerbated by EBV infection ³⁴. EBV-encoded dUTPase could enhance production of proinflammatory cytokines such as IL-6, and the tumor necrosis factor (TNF)- α by monocytes/macrophages ³⁵. Our logistic regression analysis also showed that colonic mucosal inflammation and use of corticosteroids, CyA, or TAC were associated independently with EBV infection, indicating that EBV infection or reactivation might be associated with intestinal inflammation of IBD under immunocompromised conditions similar to CMV reactivation in UC. However, it is

unclear whether the EBV detected in colonic mucosa was the primary cause of the mucosal inflammation or a secondary event to severe disease. Further basic and clinical studies will be needed.

Ten (8.3%) patients with IBD were positive for CMV, although only one was positive in the control group. The prevalence of CMV in patients with UC (13.9%) was higher than that in the control group (3.4%) and patients with CD (0%). Nine out of 10 CMV-positive patients with IBD had UC with inflamed colonic mucosa. Indeed, some investigators also reported that CMV infection was seen infrequently (0–3.6%) in colonic mucosa of patients with CD ^{32,36,37}. As for UC, CMV infection has been found more frequently in active patients (20–60%) ³⁸⁻⁴², although inactive patients do not exhibit any evidence of CMV infection ^{41,43}. The pathogenesis of CD T helper cell (Th)-1-shifted activation of cellular immunity, including IFN- γ production plays an important role in the pathogenesis of CD, and this IFN- γ could suppress CMV ⁴⁴. In contrast, TNF- α was reported to be associated with CMV activation in patients with UC. ⁴⁵. This is considered as one of the reasons why CMV reactivation is more frequently observed in patients with UC than those with CD ⁴⁶. The present study showed that older age and use of corticosteroids had the tendency to increase the risk of CMV

infection, which is consistent with findings from several previous studies ^{41,46-48}. Some basic research has shown that steroids and TNF could enhance CMV replication ⁴⁹⁻⁵¹; therefore, persistent and uncontrolled inflammation might cause the reactivation of CMV, particularly in patients who are on corticosteroids treatment.

Only a few cases of HHV-6 associated colitis have been reported ^{52,53}, although HHV-6 reactivation can cause encephalitis in the immunocompromised patients. In the present study, HHV-6 was detected in 9.2% (11/120 cases) of the patients with IBD; however, there was no difference in the prevalence among patients with UC, patients with CD, and the healthy controls. Moreover, no significant risk factor associated with HHV-6 infection was found in our logistic regression analysis. These indicate that HHV-6 infection in the colon is not related to mucosal inflammation in IBD.

The pattern of coexistence of CMV and EBV or HHV-6 in colonic mucosa is another intriguing finding of the present study. Five of 11 (45%) and one of 11 (9%) UC patients with CMV are coexisting with EBV and HHV-6, respectively. Moreover, all six patients with combined infection were those with UC. HHV-6 could be a co-factor for the development of CMV infection in patients undergoing transplantation ^{54,55}. Likewise, Aalto et al. demonstrated EBV reactivation in a large proportion of patients

with CMV primary infection ⁵⁶. These findings suggest that such interactions between CMV and other human herpes viruses might promote mucosal inflammation in IBD, particularly in UC. We, therefore, evaluated long-term outcome of UC patients with combined infection of CMV with EBV or HHV-6 to understand the pathogenic role of these infections in the clinical course. Multivariate analysis showed that combined infection, but not single infection, was the significant and independent prognostic factors for subsequent colectomy, implicating that EBV or HHV-6 infection could synergistically lead to exacerbation of intestinal inflammation or promote CMV reactivation in UC, resulting in higher rate of colectomy.

Our study has some limitations. First, this study is a retrospective study with a relatively small cohort. The small sample number, especially on long-term analysis for patients with UC, resulted in wide CI. Second, multiplex PCR assay used in this study was not quantitative PCR. Third, both biopsy samples under endoscopy and surgical resected specimens were analyzed, which could influence the result of PCR. Finally, our study did not include immunohistochemistry, antigenemia assay, and serological tests. Further prospective studies are needed to clarify these problems and validate the present findings.

In summary, we demonstrated that the prevalence of CMV and EBV in the patients with UC was higher than that in healthy controls and patients with CD using multiplex PCR method. Mucosal inflammation was associated with the risk of EBV infection. The coexistence of CMV and EBV/HHV-6 infections was associated with the subsequent colectomy in patients with UC, suggesting a synergistic role of these viruses in contributing to trigger or exacerbate the mucosal inflammation in UC.

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Tables

TABLE 1. IBD Patient Demographic and Disease Characteristics

	Controls n = 29	Ulcerative colitis n = 66	Crohn's disease n = 54
Age, median (interquartile range)	64.9 (58.0–73.0)	48.3 (34.5–60.8)	36.9 (28.3–42.0)
Gender (male / female)	17 / 12	37 / 29	42 / 12
Ulcerative colitis			
Extent of disease			
Proctitis		4	
Left-sided colitis		17	
Pancolitis		45	
Crohn's disease			
Disease location			
Ileum only			13
Colon only			7
Ileum and colon			34
Disease behavior			
Non-stricturing non-penetrating			13
Stricturing			23
Penetrating			17
Mucosal inflammation (+ / –)			
MES (Ulcerative colitis)		36 / 30	14 / 40
0 / 1 / 2 / 3		30 / 5 / 17 / 14	
Medication, n (%)			
Mesalamine		54 (81.8)	41 (75.9)
Corticosteroids		20 (30.3)	2 (3.7)
AZA or 6-MP		8 (12.1)	19 (35.2)
CyA or TAC		6 (6.8)	0 (0.0)
IFX		1 (1.5)	20 (37.0)

AZA, azathioprine; 6-MP, 6-mercaptopurine; CyA, cyclosporine A; TAC, tacrolimus; IFX, infliximab; MES, Mayo endoscopic subscore

TABLE 2. Primers used in the multiplex PCR

Virus	Sequence (5'-3')	Position	Amplicon size (bp)
HSV-1/2	F: GCCAAGAAAAAGTACATCGGCGTCATC	HSV-1: 3389	292
		HSV-2: 3058	
	R: TGAGGACAAAGTCCTGGATGTCCCTCT	HSV-1: 3680	
		HSV-2: 3349	
VZV	F: TCCGACATGCAGTCAATTTCAACGTC	49651	161
	R: GGTCGGGTAGACGCTACCACTCGTTT	49811	
EBV	F: CTTAGAATGGTGGCCGGGCTGTAAAAT	153240	229
	R: ATCCAGTACGTCTTTGTGGAGCCCAAG	153468	
CMV	F: GCGCGTACCGTTGAAAGAAAAGCATAA	80362	131
	R: TGGGCACTCGGGTCTTCATCTCTTTAC	80492	
HHV-6A/B	F: ATGCGCCATCATAATGCTCGGATACA	HHV-6A: 57837	183
		HHV-6B: 58791	
	R: CCCTGCATTCTTACGGAAGCAAAACG	HHV-6A: 58019	
		HHV-6B: 58973	
HHV-7	F: GCCCGTTTTTCGGAAATATTGGAGAGAT	55671	347
	R: ACGCACGAGACGCACTTTTCTTAAACA	56017	

HSV, herpes simplex virus; VZV, Varicella-Zoster virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus;

HHV, human herpes virus

TABLE 3. Prevalence of human herpes virus DNA in colonic mucosa

Herpes virus	controls n = 29	Ulcerative colitis n = 66	Crohn's disease n = 54	<i>P</i> value
HSV-1/2	0	0	0	
VZV	0	0	0	
EBV	0	14 / 66 (21.2%)	5 / 54 (9.3%)	0.001>
CMV	1 / 29 (3.4%)	10 / 66 (15.2%)	0	0.003
HHV-6A/B	2 / 29 (6.9%)	6 / 66 (9.1%)	5 / 54 (9.3%)	1
HHV-7	0	1 / 66 (1.5%)	2 / 54 (3.7%)	0.594

HSV, herpes simplex virus; VZV, Varicella-Zoster virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus;
HHV, human herpes virus

TABLE 4. Prevalence of human herpes viruses related to the endoscopic severity of ulcerative colitis

Herpes virus	MES 0 n = 30	MES 1 n = 5	MES 2 n = 17	MES 3 n = 14	<i>P</i> value
HSV-1/2	0	0	0	0	
VZV	0	0	0	0	
EBV	1 / 30 (3.3%)	2 / 5 (40%)	8 / 17 (47.1%)	3 / 14 (21.4%)	0.001
CMV	1 / 30 (3.3%)	0	5 / 17 (29.4%)	4 / 14 (28.6%)	0.019
HHV-6A/B	2 / 30 (6.7%)	1 / 5 (20%)	2 / 17 (11.8%)	1 / 14 (7.1%)	0.662
HHV-7	1 / 30 (3.3%)	0	0	0	1

MES, Mayo endoscopic subscore; HSV, herpes simplex virus; VZV, Varicella-Zoster virus; EBV,
Epstein-Barr virus; CMV, cytomegalovirus; HHV, human herpes virus

TABLE 5. Risk factors for EBV, CMV and HHV-6 infection in patients with ulcerative colitis and Crohn's disease: univariate analysis

	EBV		CMV		HHV-6	
	Odds ratio (95% CI)	<i>P</i> value	Odds ratio (95% CI)	<i>P</i> value	Odds ratio (95% CI)	<i>P</i> value
Age	0.99 (0.96–1.02)	0.372	1.03 (0.99–1.07)	0.120	1.01 (0.98–1.05)	0.475
Gender						
Female	Ref	–	Ref	–	Ref	–
Male	1.27 (0.452–3.56)	0.651	0.99 (0.28–3.56)	0.993	1.31 (0.38–4.47)	0.668
Disease						
Control	Ref	–	Ref	–	Ref	–
Ulcerative colitis	3.11E+07 (0–Inf)	0.989	5.00 (0.61–41)	0.134	1.35 (0.26–7.13)	0.724
Crohn's disease	1.18E+07 (0–Inf)	0.989	8.91E-08 (0–Inf)	0.991	1.38 (0.26–7.13)	0.713
Mucosal inflammation						
Noninflamed	Ref	–	Ref	–	Ref	–
Inflamed	10.20 (3.16–32.80)	0.001>	10.60 (2.20–51.40)	0.003	1.26 (0.39–4.08)	0.696
Medication						
5-ASA						
No	Ref	–	Ref	–	Ref	–
Yes	2.69 (0.96–7.51)	0.059	1.35 (0.39–4.62)	0.635	0.66 (0.21–2.13)	0.490
Corticosteroids						
No	Ref	–	Ref	–	Ref	–
Yes	3.29 (1.09–9.88)	0.034	14.30 (3.76–54.80)	0.001>	0.46 (0.06–3.70)	0.462
AZA or 6-MP						
No	Ref	–	Ref	–	Ref	–
Yes	2.13 (0.88–5.16)	0.093	0.90 (0.21–3.76)	0.881	2.14 (0.79–5.84)	0.136
CyA or TAC						
No	Ref	–	Ref	–	Ref	–
Yes	4.13 (1.700–10.10)	0.002	1.63 (0.53–5.00)	0.392	2.45 (0.99–6.04)	0.052
IFX						
No	Ref	–	Ref	–	Ref	–
Yes	1.77 (0.53–5.97)	0.356	0.59 (0.07–4.86)	0.624	0.48 (0.06–3.92)	0.496

CI, confidence interval; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HHV, human herpes virus; 5-ASA,

5-aminosalicylic acid; AZA, azathioprine; 6-MP, 6-mercaptopurine; CyA, cyclosporine A; TAC, tacrolimus; IFX, infliximab

TABLE 6. Risk factors for EBV, CMV and HHV-6 infection in patients with ulcerative colitis and Crohn's disease: multivariate analysis

	EBV		CMV		HHV-6	
	Odds ratio (95% CI)	<i>P</i> value	Odds ratio (95% CI)	<i>P</i> value	Odds ratio (95% CI)	<i>P</i> value
Age			1.06 (0.99–1.11)	0.054		
Disease						
Control			Ref	–		
Ulcerative colitis			1.54 (0.09–27.80)	0.770		
Crohn's disease			5.81E-08 (0–Inf)	0.994		
Mucosal inflammation						
Noninflamed	Ref	–	Ref	–		
Inflamed	7.56 (2.08–27.40)	0.002	6.74 (0.59–77.60)	0.126		
Medication						
5-ASA						
No	Ref	–				
Yes	2.05 (0.65–6.52)	0.223				
Corticosteroids						
No	Ref	–	Ref	–		
Yes	0.83 (0.21–3.31)	0.796	5.24 (0.91–30.30)	0.064		
AZA or 6-MP						
No	Ref	–			Ref	–
Yes	1.53 (0.56–4.18)	0.408			1.86 (0.66–5.26)	0.243
CyA or TAC						
No	Ref	–			Ref	–
Yes	2.22 (0.84–5.85)	0.106			2.23 (0.87–5.69)	0.094

CI, confidence interval; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HHV, human herpes virus; 5-ASA, 5-aminosalicylic acid; AZA, azathioprine; 6-MP, 6-mercaptopurine; CyA, cyclosporine A; TAC, tacrolimus; IFX, infliximab

TABLE 7. A multivariate Cox proportional hazard analysis of risk factor for colectomy in patient with ulcerative colitis.

	Unadjusted HR (95% CI)	<i>P</i> value	Adjusted HR (95% CI)	<i>P</i> value
Infection pattern				
Un-infection	Ref	–	Ref	–
Single-infection	0.75 (0.08–7.20)	0.802	1.39 (0.08–24.6)	0.824
Combined-infection	8.74 (1.44–53.15)	0.019	72.3 (1.85–2.82E+03)	0.022
Adjusted for partial Mayo score, Mayo endoscopic subscore, serum albumin level, serum C-reactive protein level, and anti-viral therapy				
	Unadjusted HR (95% CI)	<i>P</i> value	Adjusted HR (95% CI)	<i>P</i> value
Anti-viral therapy				
No	Ref	–	Ref	–
Yes	6.26 (0.72–54.0)	0.095	0.31 (4.13E-03–23.2)	0.595

HR, hazard ratio; CI, confidence interval

Adjusted for partial Mayo score, Mayo endoscopic subscore, serum albumin level, serum C-reactive protein level, and infection pattern

Figure Legends

FIGURE 1. Combined infection of human herpes viruses in inflammatory bowel disease (IBD)

Venn diagram representing the overlap of Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human herpes virus type 6 (HHV-6) infections. Inside of the dotted box shows the patients with ulcerative colitis (UC).