Butyric Acid in Saliva of Chronic Periodontitis Patients Induces Reactivation of EBV

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Contents

This doctoral thesis was prepared using the original article "Butyric Acid in Saliva of Chronic Periodontitis Patients Induces Transcription of the EBV Lytic Switch Activator BZLF1: A Pilot Study" (Ryo Koike, Keiko Nodomi, Norihisa Watanabe, Yorimasa Ogata, Osamu Takeichi, Masami Takei, Tadayoshi Kaneko, Morio Tonogi, Ai Kotani and Kenichi Imai. In Vivo, 2020 i*n press*) with new unpublished data (Figure 2E and Table 1).

Abstract

***Background/Aim*:** Human chronic periodontitis (CP) is a major health concern worldwide. Epstein–Barr virus (EBV) was recently reported to be associated with the progression of CP. Previous report has demonstrated that butyric acid (BA) produced by periodontopathic bacteria can inhibit histone deacetylase, thus increasing the EBV lytic switch activator *BZLF1* expression. Because higher levels of periodontopathic bacteria were detected in the saliva of CP patients, it was considered whether the saliva of CP patients can reactivate EBV. ***Materials and Methods*:** Saliva was collected from seven CP patients and five periodontally healthy individuals. BA was quantified using HPLC. Real-time PCR, luciferase assay, and western blotting were performed to determine the mRNA expression level and transcriptional activity of *BZLF1* and the phosphorylation of histone H3, respectively. ***Results*:** *BZLF1* mRNA expression and transcriptional activity were significantly higher in EBV-infected Daudi cells treated with the saliva of CP patients than with that of the healthy controls. Interestingly, although significantly higher levels of BA, propionic acid, and acetic acid were detected in the saliva of CP patients, only BA correlated significantly with the amount of *BZLF1* transcripts ($r = 0.88$; $p < 0.02$). In addition, the saliva of CP patients induced acetylation of histone H3 in Daudi cells. ***Conclusion*:** These findings suggest that BA in saliva may play a role in

the initiation of EBV reactivation and contribute to the clinical progression of EBV-related diseases, including periodontitis.

Keywords: Epstein–Barr virus; Periodontitis; butyric acid; BZLF1; reactivation

Introduction

Chronic periodontitis (CP) is a complex chronic inflammatory microbial disease that is prevalent in humans worldwide (1, 2). Severe CP can result in the loosening of teeth, occasional pain, periodontal bone loss, and eventual tooth loss (1, 2). Mounting evidence has indicated that CP is a risk factor for aspiration pneumonia, chronic obstructive pulmonary disease, diabetes, and pre-term birth (1, 2). Although no single etiological agent has been identified, a number of putative bacteria, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, are considered to be associated with CP and, thus, are used as diagnostic markers (2, 3). Recently, members of the herpes virus family, such as Epstein–Barr virus (EBV), have been suggested to be involved in the aetiology of CP because bacterial activity alone does not adequately explain the clinical characteristics of CP (4-8).

Similar to other herpes viruses, EBV establishes a persistent infection in the human host, and its life cycle has both lytic and latent phases (9, 10). The EBV-encoded immediate-early *BZLF1* gene encodes ZEBRA, a sequence-specific DNA-binding protein that is a member of the bZIP family of leucine-zipper transcriptional activators (9, 10). Since ZEBRA can transactivate early and late genes of EBV, and thereby induce the lytic cycle, this viral transcriptional activator is a master regulator of the transition from latency to the lytic

replication cycle (9, 10). EBV is frequently reactivated in immunocompromised hosts and can induce infectious mononucleosis, as well as several malignancies, such as Burkitt lymphoma and nasopharyngeal carcinoma (9-12).

Many studies have demonstrated that the amount of EBV DNA detected in the periodontal pockets and gingival tissues of CP patients is correlated with disease severity (4-8). Accordingly, Kato *et al*. previously reported that EBV DNA was more frequently detected in deep, rather than shallow, periodontal pockets among Japanese patients with CP and healthy controls (13, 14). In addition, a large number of EBV-encoded small RNA-positive B-cells were observed in the gingival tissues of CP patients (13). Although EBV is epidemiologically involved in the aetiology of CP, the process by which latent EBV is reactivated in the oral cavity remains unclear.

EBV is usually transmitted through saliva and replicates in the salivary glands, oral mucosal membrane, nasopharyngeal epithelium, and B cells (6, 7, 11, 12, 15, 16). In addition, the saliva of CP patients contains EBV-infected B cells, higher levels of EBV DNA, and greater concentrations of periodontopathic bacteria (6, 7, 15-17), suggesting a relationship between microbial interactions and the aetiology of CP. Imai *et al*. have also reported that although short-chain fatty acids (SCFAs) are secreted extracellularly by *P. gingivalis* and *F. nucleatum*, only butyric acid (BA) can induce reactivation of

EBV (18). These observations suggest that EBV reactivation may be caused by BA in the saliva of CP patients. However, no studies have yet to evaluate the amount of BA in the saliva of CP patients and determine whether the saliva can reactivate EBV.

Therefore, the aim of the present study was to assess the levels of BA in the saliva of CP patients, which could efficiently induce the expression of the EBV lytic switch activator *BZLF*1, and to determine for the first time whether there is a possible pathophysiological link with EBV reactivation. In addition, this article discusses how this relationship may pertain to the aetiology of CP.

Materials and Methods

Study approval and participants

The study protocol was approved by the Institutional Internal Review and Ethics Board of the Nihon University School of Dentistry (Tokyo, Japan; approval no. EP17D006) and conducted in accordance with the tenets of the Declaration of Helsinki. The study cohort included seven CP patients (mean age, 53.1 ± 13.7 years) and five periodontal healthy individuals (mean age, 32.6 ± 6.1 years). Written informed consent was obtained from each study participant after all procedures had been fully explained.

Reagents

BA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Antibodies (Abs) against ZEBRA and β-actin were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), whereas those against acetylated histone 3 were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and those against non-acetylated histone 3 were purchased from Abcam (Cambridge, UK).

Saliva collection

All of the study participants received dental care at Nihon University School of Dentistry. Periodontal status was assessed based on the probing pocket depth (PPD), clinical attachment level (CAL), and bleeding on probing. The PPD and CAL were measured using a PCP11 probe (Hu-Friedy Mfg. Co., LLC, Chicago, IL, USA). CP was

defined as the presence of at least two sites with a PPD of ≥5 mm and attachment loss of >6 mm.

The healthy controls had no clinical signs of gingivitis, attachment loss, or detectable bone loss on radiographic examinations, and PPD was ≤3 mm. All patients were systemically healthy with no history of periodontal treatment or any type of antibiotic therapy for at least 3 months prior to participation in this study. About 10 ml of saliva were collected from each participant. After centrifugation to remove cells and debris, the supernatant of the collected saliva was sterilised by passing through a 0.22-μm pore filter membrane and then either immediately analysed or stored at –80°C for future use.

Cell culture

Daudi cells, which are well-characterised EBV-positive human Burkitt lymphoma-derived cells, and B95-8-221 Luc cells (18, 19), which were stably transfected with the *BZLF1* promoter, were maintained at 37°C in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich Corporation, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Corporation), penicillin (100 U/ml), and streptomycin (100 μg/ml). For the stimulation experiments, cells (1.0×10^6 cells /1.0-ml well) were treated with saliva or BA.

Quantification of SCFAs

SCFAs [BA, propionic acid (PA), acetic acid (AA), isoBA, and isovaleric acid] were quantified using ion exclusion high-performance liquid chromatography (HPLC), as described previously (18, 20). Briefly, each saliva sample was mixed with 12% perchloric acid, filtered through a cellulose acetate membrane filter (Cosmonice Filter W, pore size: 0.45 μm; Nacalai Tesque, Inc., Kyoto, Japan), and then injected into a SIL-10 auto injector (Shimadzu Corporation, Kyoto, Japan). SCFAs were separated using a serial organic acid column and a guard column with isocratic elution of p-toluene sulfonic acid aqueous solution and detected using an electronic conductivity detector.

Preparation of mRNA and real-time polymerase chain reaction (PCR)

The experimental procedures for RNA purification and real-time PCR were performed as previously described (18, 21). Briefly, Daudi cells were washed once with ice-cold phosphate-buffered saline (PBS) and homogenised using a QIAshredder (QIAGEN, Alameda, CA, USA), while total RNA was purified using an RNeasy Mini Kit (QIAGEN). For cDNA synthesis, total RNA (1 μg) was reverse transcribed using an RNA PCR kit (PrimeScript; Takara Bio, Shiga, Japan). The resulting cDNA mixture was subjected to real-time PCR analysis using SYBR Premix Ex *Taq* solution (Takara

Bio) containing 5 μM sense and antisense primers. The primer sequences used for the amplification of each gene were as follows: *BZLF1* forward (5-TTC CAC AGC CTG CAC CAG TG-3) and reverse (5- GGC AGC AGC CAC CTC ACG GT -3); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), forward (5-ACC AGC CCC AGC AAG AGC ACA AG-3) and reverse (5-TTC AAG GGG TCT ACA TGG CAA CTG-3). PCR assays were performed using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) and analysed using the software provided by the device manufacturer. The thermal cycling conditions were 40 cycles at 95°C for 5 sec, 60°C for 30 sec, and 72°C for 1 min. All real-time PCR experiments were performed in triplicates, and the specificity of each product was verified *via* a melting curve analysis. The calculated gene expression levels were normalised to *GAPDH* mRNA levels.

Luciferase assay

Luciferase assay was then performed using a Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. The experimental procedure for the luciferase assay has been previously reported (20, 21). B95-8-221 Luc. cells were harvested and the extracts were subjected to luciferase assay using the Dual-Luciferase Assay System™ (Promega). All the experiments were carried out in

triplicates and the data were presented as the fold increase in luciferase activities (means ± S.D.) relative to the control of three independent transfections.

Immunoblot assay

The experimental procedures for immunoprecipitation and immunoblotting were performed according to previously published protocols (22, 23). Briefly, cells were harvested with lysis buffer (25 mM HEPES-NaOH [pH 7.9], 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3 % NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), the proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, MA, USA). The protein content was measured by a detergent-compatible protein assay kit (Bio-Rad, Hercules, Hercules, CA, USA). All membranes were treated with ECL prime detection reagent (Thermo Fisher Scientific) prior to examination. All bands were visualised using a ChemiDoc XRS System (Bio-Rad).

Statistical analysis

Comparison of two groups was performed using the two-tailed Student's *t*-test. The correlation coefficient (r) was calculated where applicable. A probability (*p*) value of <0.05 was considered statistically significant.

Results

Saliva of CP patients contains relatively high levels of SCFAs

Previous studies have reported that the periodontal pockets and dental plaques of CP patients contain high concentrations (mM levels) of SCFAs (24-26). However, the amounts of SCFAs have not been investigated in the saliva of Japanese CP patients. Therefore, this study measured the concentrations of SCFAs in the saliva of seven CP patients and five healthy controls by HPLC. As presented in Figure 1, the saliva of CP patients contained significantly higher levels ($p < 0.01$) of BA, PA, and AA. On the other hand, the amounts of isoBA and isovaleric acid in the saliva were very low. The concentrations of BA, PA, and AA in the saliva of CP patients were 0.31–1.37, 0.49–1.35, and 2.12–5.81 mM, respectively. On average, the saliva of CP patients contained about 33.3-, 3.3-, and 2.4-fold higher levels of BA, PA, and AA than that of the healthy controls. The concentration of SCFAs in the saliva of each individuals were shown Table1.

Saliva of CP patients induces expression of *BZLF1*

Since high concentrations of SCFAs were found in the saliva of CP patients, whether the saliva can reactivate EBV was investigated. Real time-PCR was conducted to evaluate the effect of the saliva of seven CP patients and five healthy controls at a 1:2 dilution on *BZLF1* mRNA expression in Daudi cells. As presented in Figure 2A, mRNA levels of the EBV lytic

gene *BZLF1* were significantly higher in cells treated with the saliva of CP patients than of that of the healthy controls. Interestingly, there was a significant correlation between BA concentrations and *BZLF1* transcript levels (r=0.88; $p<0.02$; Figure 2B). The concentration of BA in the saliva of CP patients (0.31–1.37 mM) induced *BZLF1* expression in a concentration-dependent manner (Figure 2C). In contrast, no such effect was observed with PA and AA (data not shown). Next, the effect of saliva on gene expression of the *BZLF1* promoter was examined using the luciferase assay. As demonstrated in the results presented in Figure 2D, the saliva of CP patients transactivated the *BZLF1* promoter in B95-8-221 Luc cells. In addition, although no such effect was observed with PA and AA (data not shown), the concentration of BA in the saliva of CP patients transactivated the *BZLF1* promoter in B95-8-221 Luc cells. (Figure 2E).

Saliva of CP patients induces expression of ZEBRA

The expression of the lytic switch transactivator ZEBRA by exposure to the saliva of CP patients was examined. As presented in Figure 3, the saliva of the healthy controls had no effect on the expression of ZEBRA in Daudi cells. However, the addition of the saliva of CP patients increased the expression of ZEBRA.

Hyperacetylation of histones by the saliva of CP patients

BA is known to inhibit the enzymatic activity of histone deacetylase (HDAC) by competing with the HDAC substrate for the enzyme's active site pocket, which contains the catalytic center (27), thus stimulating transcription of various genes, including *BZLF1* (9, 10, 28). Imai *et al*. have previously demonstrated that the culture supernatant from periodontopathic bacteria, which contains high concentrations of BA, can inhibit HDACs, thereby increasing the level of histone acetylation and the transcriptional activity of the *BZLF1* gene (18). Next, the effects of saliva and BA on histone acetylation were examined by western blotting with Abs specific for acetylated histone H3. As presented in Figure 4, although there was no effect by the saliva of the healthy controls, both the saliva of CP patients and BA induced acetylation of histone H3 (Figure 4A, B). In contrast, no such effect was observed with the other tested SCFAs (data not shown).

Discussion

Reactivation of latent EBV is associated with progeny virus production and several human diseases (9-12). Therefore, elucidation of the mechanisms that promote or disrupt EBV latency in infected individuals is required to understand the pathobiology of EBV infection and to develop preventive measures and novel therapies. However, the trigger that is responsible for the switch from latency to the lytic cycle in individuals latently infected with EBV remains unclear. This study examined the biological actions of the saliva of CP patients and healthy controls on the reactivation of EBV infection.

It has been reported that more than sufficient concentrations of BA are present in the dental plaques (range= 4.7–13.8 mM) (25, 26) and periodontal pockets (mean, 2.6 ± 0.4 mM) of patients with periodontal disease (24), whereas the BA concentration is below the detection limits in healthy sites, suggesting that BA may play a role in the initiation of EBV reactivation and contribute to the clinical progression of EBV-related diseases. The results of the present study revealed significantly higher levels of BA in the saliva of CP patients, which could efficiently induce *BZLF1* transcription. These observations suggest that the BA content in the saliva of CP patients might be involved in the progression of EBV-related diseases as well as periodontitis.

Since *BZLF1* expression is a key step in the reactivation from latency in EBV-infected cells (9, 10), this study focused on the expression of this transcriptional activator. Although, neither *BZLF1* mRNA nor ZEBRA protein was detectable during latency, high levels of anti-BZLF1 Abs in blood are associated with an increased EBV serum load in EBV-infected patients (29). Figure 1 shows that high concentrations (mM levels) of BA, AA, and PA are present in the saliva of Japanese CP patients (Figure 1). Interestingly, there was a significant correlation between BA concentrations and the levels of *BZLF1* transcripts ($r=0.88$; $p<0.02$; Figure 2B). In fact, BA was the only acid in the saliva of CP patients that induced BZLF1 expression (Figure 2C). These results support the findings of a previous *in vitro* study, which reported that among several SCFAs in the culture supernatant of periodontopathic bacteria, only butyrate reactivated EBV, whereas non-butyrate-producing bacteria did not (18).

Recent studies have revealed a new mechanism that regulates the maintenance and reversal of EBV latency, which involves nucleosome configurations and histone modifications. In the latent state, the *BZLF1* gene promoter is bound by histone proteins into a chromatin structure that serves to repress the transcription of *BZLF1* (9, 10). Hyperacetylation of core histone proteins adjacent to the *BZLF1* promoter was correlated with transcriptional activation of *BZLF1*, whereas hypoacetylation mediated by HDAC was

correlated with its repression, which is considered responsible for the maintenance of latency (9, 10). Since BA is one of the most potent inhibitors of HDACs and previous report indicated that BA in the culture supernatant of periodontopathic bacteria promotes histone acetylation and the transcriptional activity of the *BZLF1* gene (18), in this study whether the saliva of CP patients induced histone acetylation was examined. Figure 4 shows that the saliva of CP patients induced Lys acetylation of histone H3 in EBV-infected cells. Imai *et al.* have reported that no such activity occurred with *P. gingivalis* or bacterial components, such as lipopolysaccharide and fimbriae (16). Although it is necessary to assess other factors contained in saliva, such as cytokines and enzymes, these findings suggested that H3 histone acetylation and BZLF1 expression are ascribable to BA contained in the saliva of CP patients.

The saliva of patients with periodontitis contains EBV-infected B cells, and bleeding of the gums is often observed in these patients (7, 15-17). In addition, it was recently reported that EBV infects the oral epithelial cells of patients with periodontitis in addition to the epithelial cells of the upper aerodigestive tract (30). The extent of gingival epithelial EBV infection is correlated with the severity of CP (30). Moreover, previous reports, as well as the present study, indicated that EBV also contributes to the progression of periapical periodontitis (20, 31). These findings and previous observations suggest the potential risks of BA in saliva for the progression of

periodontitis and periapical periodontitis. In this study it was assumed that microbial synergy by the interaction between periodontopathic bacteria and EBV leads to the following negative chain of pathological events in the oral cavity: 1) periodontopathic anaerobic bacteria, such as *P. gingivalis* and *F. nucleatum*, produce BA; 2) BA induces EBV reactivation; 3) EBV impairs local host defences, 4) which leads to increased proliferation of periodontopathic bacteria; 5) increased BA and inflammatory cytokine production by the synergistic effects of EBV and periodontopathic bacteria; and 6) periodontitis escalation.

Periodontitis and EBV are spreading worldwide. Although the findings of this study suggest a relationship between the saliva of patients with periodontitis and EBV reactivation, additional basic and clinical studies with greater numbers of cases are needed. Furthermore, prevention and early treatment of periodontitis involving elimination of BA-producing bacteria could effectively block further clinical progression of EBV infection.

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	SCFA (Short-Chain Fatty Acid Level) (mM)				
	Butyric acid	Propionic acid	Acetic acid	Isobutyric acid	Isovaleric acid
HC 1	0.02	0.14	0.91	0.01	0.01
HC 2	0.04	0.30	2.35	0.06	0.03
HC 3	0.07	0.23	1.59	0.02	0.01
HC 4	0.06	0.47	2.44	0.02	0.01
HC 5	0.01	0.05	0.73	0.01	0.01
CP 1	0.52	0.69	2.46	0.02	n.d
CP 2	1.04	0.49	3.48	0.04	0.02
CP 3	1.19	0.49	2.12	0.01	0.01
CP 4	1.37	0.52	2.86	0.09	0.05
CP 5	0.31	0.65	3.65	0.01	0.01
CP 6	1.20	0.49	3.52	0.04	0.02
CP 7	1.04	1.35	5.81	0.02	0.02

Table 1. Concentration of SCFAs in the saliva of each individuals

HC: healthy control; CP: chronic periodontitis

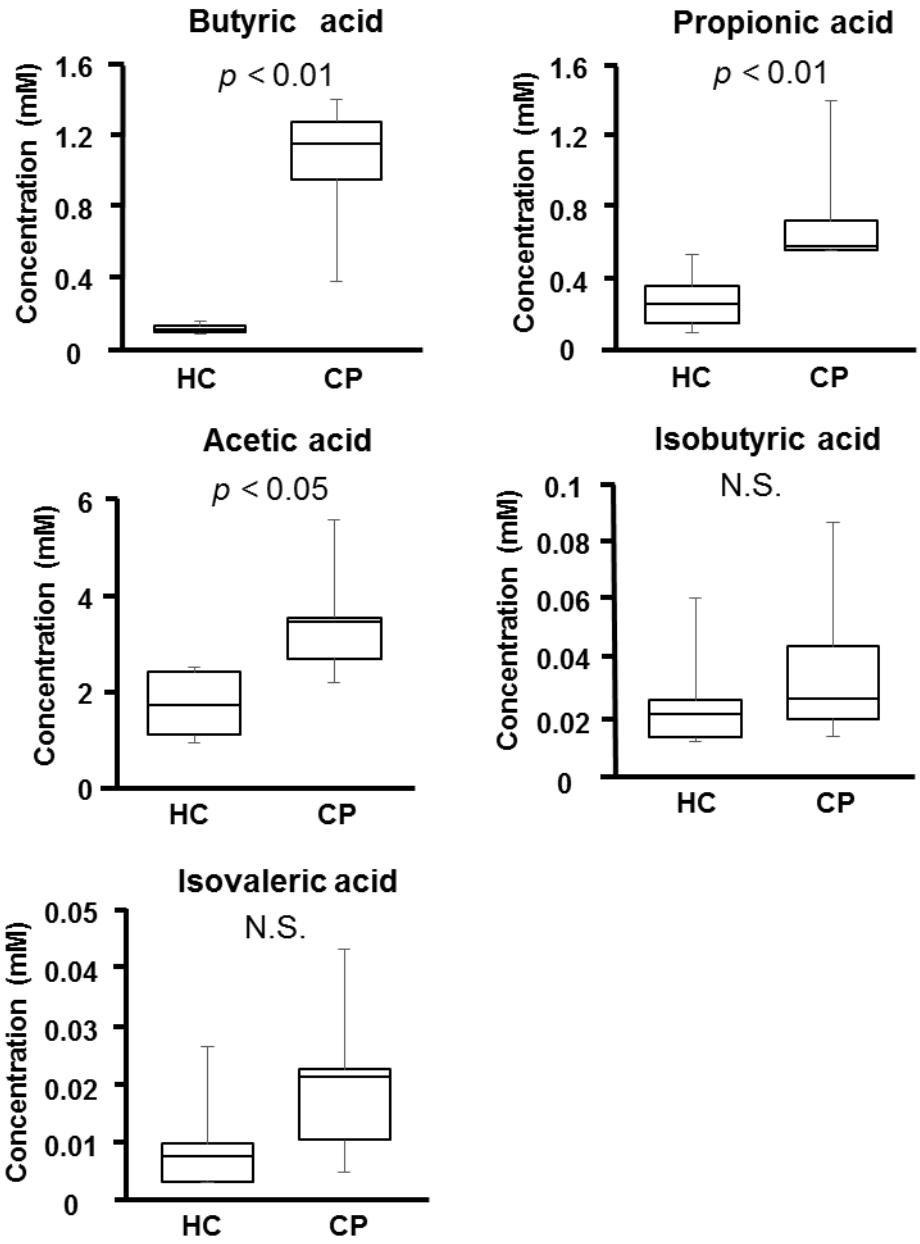

Figure 1. Saliva of CP patients contains higher levels of SCFAs.

Levels of of butyric acid, propionic acid, acetic acid, isobutyric acid, and isovaleric acid in the saliva of seven CP patients and five healthy controls. HC: healthy control; CP: chronic periodontitis; N.S.: not significant.

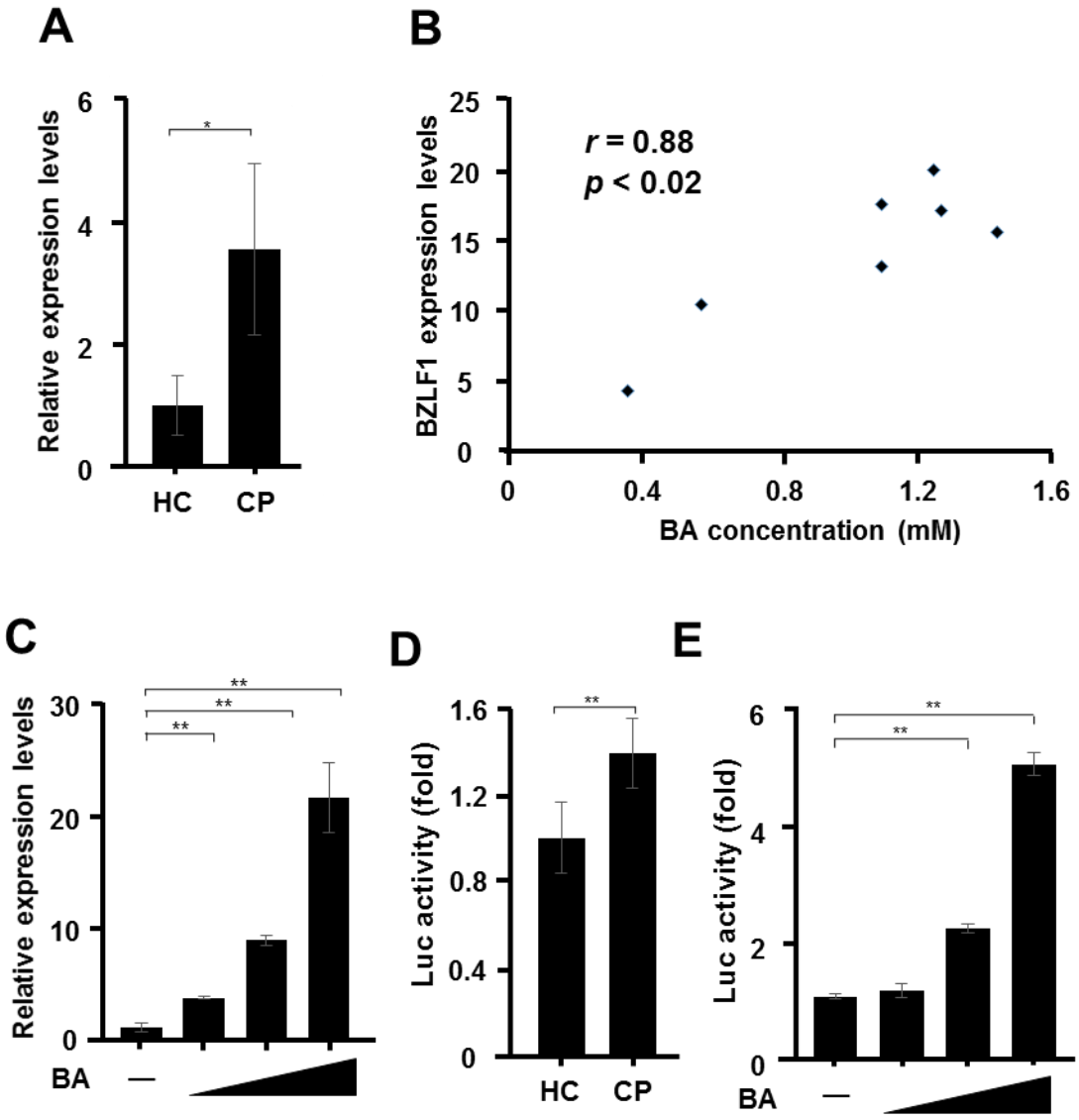

Figure 2. Induction of *BZLF1* gene expression by the saliva of CP patients.
(A) Daudi cells were incubated with the saliva of seven CP patients and five healthy controls at a 1:2 dilution (saliva volume *vs*. total cell culture medium volume) for 24 h. Real-time PCR analysis was conducted with specific primers to detect *BZLF1* mRNA expression. (B) The correlation coefficient (r) was calculated between butyric acid (BA) concentrations and *BZLF1* mRNA levels. (C) Daudi cells were treated with BA (0.5, 1.0, or 1.5 mM) for 24 h, and *BZLF1* mRNA expression was assessed. (D) B95-8-221

Luc cells were treated with the saliva of seven CP patients and five healthy controls at a 1:2 dilution for 48 h. (E) B95-8-221 Luc cells were treated with BA (0.5, 1.0, or 1.5 mM) for 48 h. The luciferase activity of each cell lysate was then measured. The values are presented as mean ± standard deviation (n = 3). **p<0.01, *p<0.05.

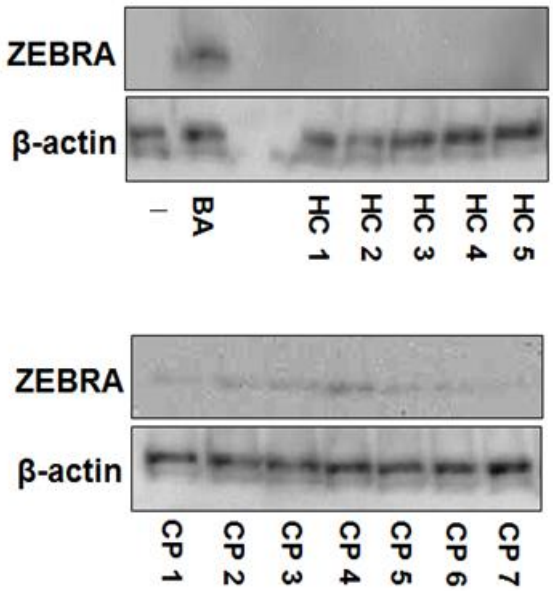

Figure 3. Saliva of CP patients induced ZEBRA expression.

Daudi cells were treated with the saliva of seven CP patients and five healthy controls at a 1:2 dilution or BA (2 mM, positive control) for 36 h. The lysates were harvested, and ZEBRA levels were assessed by immunoblotting using specific Abs. β-actin was used as an internal control. BA: butyric acid.

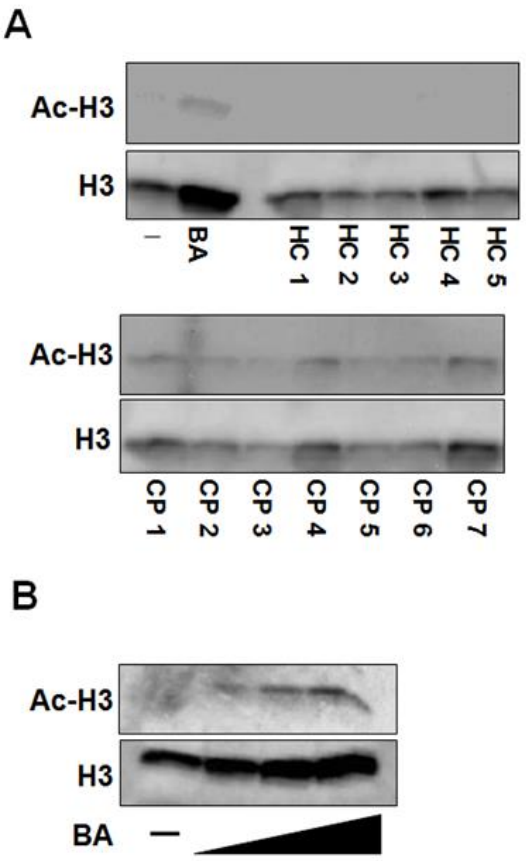

Figure 4. Hyperacetylation of histones by the saliva of CP patients.

(A) Daudi cells were treated with the saliva of seven CP patients and five healthy controls at a 1:2 dilution or BA (2 mM) for 36 h. (B) Daudi cells were treated with BA (0.5, 1.0, or 1.5 mM) for 36 h. The lysates were harvested, and acetylated histone proteins were assessed by western blot analysis using Abs against acetylated histone 3. The unmodified H3 protein was used as control. BA: butyric acid.