VP26, a herpes simplex virus type 1 capsid protein, increases DNA methylation in COASY promoter region

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**ARTICLE INFO**

**Keywords:**
Herpes simplex virus type 1
DNA methylation
COASY
VP26
Herpesviridae

**ABSTRACT**

It has been reported that some specific changes in DNA methylation can be due to aging or infection by tumor-related viruses but the effect of herpes simplex virus type 1 (HSV-1) in this regard is unknown. HSV-1 is a well-known virus that causes cold sores. After the primary infection, the virus switches to latent infection and remains in the body for the whole life. As the location of DNA methylation, we focused on the promoter region of the COASY gene, which codes for coenzyme A synthase, because methylation in this region is reportedly associated with Alzheimer’s disease (AD). During HSV-1 lytic infection, compared to non-infected cells, COASY DNA methylation decreased but when HSV-1 replication was inhibited by acyclovir, an anti-herpes agent, COASY DNA methylation increased. In addition, for expression of immediate early protein only, there was no significant change in COASY DNA methylation, while for expression of the capsid protein VP26, a late protein known to bind with DNA methyltransferase DNMT3A, in the nucleus only, COASY DNA methylation significantly increased compared to the control, without changes in DNMT3A mRNA. Our results suggested that DNA methylation occurred not due to transcriptional changes in DNMT3A but through translational regulation. In this research, we showed that host COASY DNA methylation is altered by HSV-1 infection, in particular by HSV-1 VP26. It is a potential cause of various diseases, and this is particularly relevant for AD.

1. Introduction

Infection by herpes simplex virus type 1 (HSV-1) causes cold sores and occurs worldwide (Whitley et al., 2007). While the prevalence of HSV-1 in the global population varies by region, the global prevalence between age 0 and 49 in 2016 was estimated to be 66.6% (James et al., 2020). In addition, it was previously reported that HSV-1 has a ubiquitous presence and that the infection rate can be as high as 90% (Fatahzadeh and Schwartz, 2007; Itzhaki and Wozniak, 2008). After the primary infection, it is transported retrogradely through the peripheral sensory nerves to the trigeminal ganglia. At the ganglia, HSV-1 establishes life-long latency (Preston and Efstathiou, 2007). HSV-1 is reactivated from latency by a variety of factors including sunburn, extreme temperatures, stress and fatigue, to cause cold sores again. However, during the long clinically asymptomatic period, the effect of HSV-1 on the host is unknown.

Previously, it was assumed that there was a causal relationship between HSV-1 infection of the central nervous system (CNS) and Alzheimer’s disease (AD) (Itzhaki et al., 1997; Steiner et al., 2007). HSV-1 is a neurotropic virus and HSV-1 DNA and RNA were detected at higher rates in the postmortem brains of AD patients as compared with non-AD elderly subjects (Zhang et al., 2022). However, as the amounts were extremely small, there is presently insufficient evidence to implicate HSV-1 as a cause in the pathogenesis of AD.

However, serologically, it was reported that HSV-1 antibody levels were higher in AD patients than in normal elderly controls (Katan et al., 2013; Letenneur et al., 2008) and avidity, an indicator of reactivation, was also higher (Kobayashi et al., 2013; Mancuso et al., 2014). While associations of HSV-1 and AD have been much reported, a conclusion remains elusive.

If HSV-1 enters the CNS and proliferates, it causes severe encephalitis, though the route of entry has still to be fully elucidated (Menendez and Carr, 2017; Steiner et al., 2007). However, active replication of HSV-1 as in encephalitis is not observed in the AD brain so if it is present, it is in the latent state. Therefore, we considered that HSV-1 could influence epigenetic regulation in the brain and be involved in the onset of AD through changes in gene expression over many years, even with a small quantity of virus. During infection, when HSV-1 invades cells and proliferates in them, virus proteins are expressed in the order immediate early (IE), early (E), late (L) protein (Gruffat et al., 2016). Since the HSV-1 genome is transported within capsids in axons (Smith et al.,...
2004), the virus may be present in capsid form when it invades the brain and still affect it in some way despite the absence of proliferation.

The mechanism of HSV-1 reactivation remains unclear, but there is increasing evidence that latency is epigenetically regulated (Kennedy et al., 2015). It was previously reported that in epigenetic control for HSV-1 genes, latent gene expression is not controlled by DNA methylation but by histone modifications (Bloom et al., 2010). On the other hand, not much is known about epigenetic control of host genes by HSV-1 infection.

DNA methylation, a known epigenetic mechanism, affects gene expression and phenotypes without any changes in gene sequences (Feil and Fraga, 2012). DNA methylation is associated with multiple diseases, in particular cancer and AD, whose risk increases with aging (Xie et al., 2022; Zabransky et al., 2022). DNA methylation changes with aging (Hannum et al., 2013; Horvath, 2013), as well as due to environmental factors such as chemical exposure (Martin and Fry, 2018). It can also be altered through infection by tumor-related viruses such as polyomavirus, adenovirus, papillomavirus, Epstein-Barr virus (EBV) and Kaposi’s sarcoma herpes virus (KSHV) (Milavetz and Balakrishnan, 2015). However, a detailed molecular mechanism for changes in DNA methylation remains unclear and it is not known whether non-tumor-related viruses cause DNA methylation. Therefore, in the present study, we investigated whether HSV-1, a virus that does not cause tumors, is associated with DNA methylation.

DNA methyltransferases (DNMTs) are enzymes that cause DNA methylation and there are 5 known types - DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. Among them, DNMT2 and DNMT3L have no DNA methylation activity despite their names (Fuks et al., 2003; Goll et al., 2006). DNMT1 functions during DNA replication to copy the methylation pattern of the original strand to the newly synthesized one. DNMT3A and DNMT3B, however, are involved in the synthesis of new methylation patterns (Moore et al., 2013). Therefore, there is a strong possibility that factors binding to DNMT1, DNMT3A and DNMT3B are associated with changes in DNA methylation. It was reported that viral protein (VP) 26, an HSV-1 capsid protein, associates and colocalizes with DNMT3A (Rowles et al., 2015). Known to be the smallest capsid protein, VP26 is encoded by the UL35 gene and composed of 112 amino acids (McGeoch et al., 1988). While VP26 is not essential for capsid assembly, its phosphorylation is thought to prevent aggregation of HSV-1 capsid proteins and aid in efficient virus replication (Kobayashi et al., 2015). However, it was previously unknown whether VP26 had any influence on changes in host DNA methylation.

Many studies, including one by the authors, reported the occurrence of DNA methylation in AD (Kobayashi et al., 2016a; Qazi et al., 2018; Xie et al., 2022), but its cause has been unclear up till now. We previously demonstrated that blood DNA methylation levels in the promoter region of COASY, the gene that encodes coenzyme A synthase, were higher in AD than in normal controls (Kobayashi et al., 2016b, 2020). As another previous study demonstrated similar alterations in DNA methylation in the blood and in the brain (Uno et al., 2016), we speculated that changes in blood DNA methylation would reflect changes in brain DNA methylation levels. In view of the above, in the present study, we investigated the effects of HSV-1 infection on DNA methylation in the COASY gene promoter region.

We also examined the effect of VP26, the capsid protein that binds to DNMT3A, by itself. As VP26 does not have a nuclear localization signal (NLS), VP5 and VP19C are needed for it to migrate into the nucleus (Homa and Brown, 1997; Rixon et al., 1996). Therefore, in the present study, we enabled VP26 alone to be expressed in the nucleus by adding 3 copies of the NLS of the virus of large T-antigen, fused to the C terminus (Kalderon et al., 1984; Lanford et al., 1986).

2. Materials and methods

2.1. Cells, cell culture and virus

The Human neuroblastoma cell line SH-SY5Y was purchased from European Collection of Authenticated Cell Cultures (ECACC). The cells were maintained at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific), supplemented with 15% heat inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 10 µg/ml streptomycin. The HSV-1 KOS strain was purchased from American Type Culture Collection (ATCC).

2.2. HSV-1 infection

SH-SY5Y cells were infected by the HSV-1 KOS strain at multiplicity of infection (MOI) of 0.25 for an hour. After infection, the medium was replaced with fresh medium. The same experiment was carried out on 6 well plates and 8 well chamber slides (Thermo Fisher Scientific). Cells were collected 24 h after infection.

2.3. HSV-1 infection with acyclovir

SH-SY5Y cells were pretreated with 150 µg/mL of the anti-herpes drug acyclovir (ACV, Merck) for a day (Millin et al., 1988). They were then infected with the HSV-1 KOS strain at multiplicity of infection (MOI) 5 in the continued presence of ACV for an hour. Next, the medium with the virus was replaced with fresh medium containing 150 µg/mL of ACV. The same experiment was carried out on 6 well plates and 8 well chamber slides. Cells were collected 24 h after infection.
2.4. Expression of HSV-1 immediate early protein alone

SH-SY5Y cells were pretreated with 50 μg/mL of the protein synthesis inhibitor cycloheximide (CHX, Merck) for an hour and then infected by the HSV-1 KOS strain at MOI 5 in the continued presence of CHX for an hour. Next, the medium containing the virus was replaced with fresh medium containing CHX. Six hours after infection, some cells were collected as control samples. The medium with CHX was replaced with fresh medium containing either 1 μg/mL of the RNA synthesis inhibitor actinomycin D (Act-D, Merck) or 100 μM of the cyclin-dependent kinase (cdk) inhibitor Roscovitine (Rosc, Merck) (Davido et al., 2002). Rosco inhibited HSV-1 replication, transcription of IE and E genes, and viral DNA synthesis (Schanig et al., 1999) (Fig. 2A). Twenty-four hours after infection, cells were collected.

2.5. VP26 plasmid construction and transfection

Using HSV-1 KOS DNA as a template, UL35, with 2 restriction enzyme recognition sequences (for BglII and Kpn I) added, was amplified by PCR. 50 μL of reaction mixtures consisted of 1 μL of KOD FX Neo (TOYOBO), 25 μL of 2 × PCR buffer for KOD FX Neo (TOYOBO), 10 μL of 2 mM dNTP, 0.2 μL each of 50 μM primer sets (5′-TCCAGATCTCCGGTCCCGGCAAATG-3′ and 5′-TCTGTGACACGCGGTC CGGGGGCTGAAAGGTATCG-3′), 1 μL of template DNA, and 12.6 μL of H2O. PCR conditions were an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 68 °C for 30 s. Using a standard technique, UL35 and pEGFP-N1 vector (Clontech) expressing enhanced green fluorescent protein (EGFP) were digested with BglII and Kpn I and a new vector pVP26-EGFP was created by inserting UL35 into pEGFP-N1. Next, single-stranded DNA oligonucleotides including three copies of the NLS (GATCCAAAAAGAAGAGAAAGGTATCG-3′) of the simian virus 40 large T-antigen, with 2 restriction enzyme recognition sequences (for HindII and Kpn I) added, was synthesized by PCR. 50 μL of a solution consisting of Tris buffered saline with protein (1:100; ab110226, Abcam), ICP8 E protein (1:50; ab20194, Abcam), and VP16 L protein (1:100; ab110226, Abcam) were used as primary antibodies. They were diluted with a solution consisting of Tris buffered saline with Tween 20 (TBST, 100 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20), 1% bovine serum albumin (BSA), and Na2SO added to wells and incubated at 37 °C for 1 h. Slides were then washed 3 times in PBS with Tween 20 (PBST). 488 anti-goat mouse IgG (1:200; Thermo Fisher Scientific) was added as a secondary antibody followed by incubation for 30 min at 37 °C in the dark and washing twice in PBST. Slides were observed using the BX51 fluorescence microscope (Olympus).

2.6. DNA methylation analysis

DNA and RNA were extracted from collected cells using the AllPrep DNA/RNA Mini Kit (Qiagen). 500 ng - 1 μg of each DNA sample was bisulfite converted using an EpiTect Plus DNA Bisulfite Kit (Qiagen). Methylated and unmethylated DNA were created as previously stated (Kobayashi et al., 2020). EpiScope unmethylated HCT116 double knockout (DKO) genomic DNA (Takara Bio) was used as unmethylated human genomic DNA, and methylated DNA was generated by methylating at high levels using CpG methylation. A calibration curve, including methylation rates of 100%, 75%, 50%, 25%, 5%, and 0%, was established by mixing bisulfite unmethylated and methylated DNA samples at different rates. Using the calibration curve, methylation levels of the bisulfited samples in the COASY promoter region were measured by methylation-sensitive high-resolution melting (MS-HRM) as previously stated (Kobayashi et al., 2020). 0.6 μL of the bisulfited DNA samples was amplified in 20 μL reaction mixtures, the remainder consisting of 10 μL of 2x MeltDoctor HRM Master Mix (Thermo Fisher Scientific), 0.12 μL each of 50 μM forward and reverse primers, and 9.16 μL of H2O. PCR conditions were an initial denaturation step of 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 60 s, using QuantStudio 3 (Thermo Fisher Scientific). Then, MS-HRM was performed to quantify methylation levels of the samples by applying the results to the calibration curve. For the analyses, Design and Analysis Software v1.4.3 (Thermo Fisher Scientific) and High Resolution Melt Software v3.1 (Thermo Fisher Scientific) were used.

2.7. Fluorescent immunostaining

For the immunofluorescent staining, cells were washed with phosphate-buffered saline (PBS) and fixed with methanol/acetic acid. Anti-HSV-1 antibodies for infected cell protein (ICP) 4 IE protein (1:200; ab6514, Abcam), ICP8 E protein (1:50; ab20194, Abcam), and VP16 L protein (1:100; ab110226, Abcam) were used as primary antibodies. They were diluted with a solution consisting of Tris buffered saline with Tween 20 (TBST, 100 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20), 1% bovine serum albumin (BSA), and Na2SO added to wells and incubated at 37 °C for 1 h. Slides were then washed 3 times in PBS with Tween 20 (PBST). 488 anti-goat mouse IgG (1:200; Thermo Fisher Scientific) was added as a secondary antibody followed by incubation for 30 min at 37 °C in the dark and washing twice in PBST. Slides were observed using the BX51 fluorescence microscope (Olympus).

2.8. Real-time RT-PCR

The mRNA levels of DNMT1, DNMT3A, DNMT3B and β-actin (ACTB), as an endogenous control, were measured using real-time RT-PCR. Briefly, 500 ng of RNA samples were converted to complementary DNA (cDNA) using the PrimeScript RT reagent Kit (Takara Bio) in 10 μL of reaction volume. Next, reactions were performed in duplicate with a total volume of 25 μL of a solution consisting of 12.5 μL of Premix EX Taq (Perfect Real Time, Takara Bio), 0.225 μL each of 100 μM forward and reverse primers, 0.625 μL of 10 μM TaqMan probe, 0.5 μL of ROX Reference Dye, 8925 μL of H2O, and 2 μL of cDNA samples. The thermal profile was an initial denaturation step of 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 60 s, using QuantStudio 3 (Thermo Fisher Scientific). Primer sequences were as follows: Forward primer (5′-AGGGACCATCATCGTGAAGACA-3′), reverse primer (5′-CTGACCCCTTGGGCAAGG-3′), and probe (5′-FAM-CATGCGGGCAGCCACAATG-CATC-TAMRA-3′) were used for DNMT1 mRNA; forward primer (5′- AGGGACCATCATCGTGAAGACA-3′), reverse primer (5′-CTGACCCCTTGGGCAAGG-3′), and probe (5′-FAM-CATGCGGGCAGCCACAATG-CATC-TAMRA-3′) were used for DNMT3A mRNA; forward primer (5′- CGGTGGGTGCATGGTGTGTTG-3′), reverse primer (5′-AGGAAGCAGGAGTTATTGAAGGTG-3′), and probe (5′-FAM-CATGCGGGCAGCCACAATG-CATC-TAMRA-3′) were used for DNMT3B mRNA; and forward primer (5′- CCGTGGGTGCATGGTGTGTTG-3′), reverse primer (5′- AGGGACCATCATCGTGAAGACA-3′), forward primer (5′- CCTTGGGCAAGGGCCACG-3′), and probe (5′-FAM-CATGCGGGCAGCCACAATG-CATC-TAMRA-3′) were used for ACTB mRNA. For analyses, Design and Analysis Software v1.4.3 (Thermo Fisher Scientific) was used.

2.9. Statistical analysis

The Shapiro–Wilk test was used to test the normality of the data. The unpaired t-test was performed to compare DNA methylation levels between 2 groups. Multiple-group comparisons of parametric data were conducted using One-way analysis of variance (ANOVA) and of non-parametric data using the Kruskal-Wallis test. In addition, if values were significant, Tukey’s multiple comparisons test and Dunn’s multiple comparisons test were used for subsequent testing, as post hoc tests, as needed. Statistical analyses were conducted using Prism 9 (GraphPad Software).
3. Results

3.1. COASY DNA methylation levels decrease in HSV-1 lytic infection

SH-SYSY cells were infected with the HSV-1 KOS strain, which undergoes a lytic replication cycle, at MOI 0.25, which increased in a lytic manner. DNA methylation levels at the COASY promoter region (COASY DNA methylation levels) in SH-SYSY cells 24 h after infection were assumed to be normally distributed. COASY DNA methylation levels after infection with HSV-1 were significantly lower compared to mock infection (\(P = 0.030\), unpaired \(t\)-test, Fig. 1 A). In addition, among the cells that survived HSV-1 infection, expression of ICP4 as the IE protein, ICP8 as the E protein, and VP16 as the L protein were confirmed by fluorescent immunostaining (Fig. 1C).

3.2. COASY DNA methylation levels increase in HSV-1 infection with ACV

We also infected SH-SYSY cells at a higher concentration, MOI 5, adding ACV, which inhibits active replication of HSV-1. COASY DNA methylation levels for SH-SYSY cells infected with HSV-1 in the presence of ACV were assumed to be normally distributed. This time, a significant increase in COASY DNA methylation levels was observed in HSV-1 infection compared to mock infection (\(P = 0.039\), unpaired \(t\)-test, Fig. 1B). Also, ICP4, ICP8 and VP16 were expressed, though the intensity of immunofluorescence staining showed that levels of expression were

Fig. 1. COASY DNA methylation levels and viral protein expression post HSV-1 infection

Fig. 2 shows DNA methylation levels in the COASY promoter region after infection for 24 h with HSV-1 KOS at MOI 0.25 (A) or with ACV added, at MOI 5 (B) (A, mock: \(n = 6\), 24 hpi: \(n = 11\); B, mock: \(n = 9\), 24 hpi: \(n = 5\)). Columns with error bars indicate mean with standard errors of the mean (SEM). *\(P < 0.05\) by unpaired \(t\)-test. hpi: hours post infection.

(C) Upper panel shows SH-SYSY cells infected with HSV-1 KOS at MOI 0.25 and lower panel with ACV added and infected at MOI 5, under immunofluorescence microscopy. Stained green are the IE protein ICP4 in the left column, the E protein ICP8 in the center column and the L protein VP16 in the right column. Nuclei are stained with DAPI (blue). Scale bar: 50 \(\mu\)m. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
lower with ACV than without ACV (Fig. 1C).

3.3. No significant changes in COASY DNA methylation levels for expression of IE protein

With HSV-1 infection, COASY DNA methylation levels for SH-SY5Y cells expressing IE protein only were assumed to be normally distributed, this including the control. When Act. D and Rosco were used to stop the cascade beyond E genes, there were no significant changes in COASY DNA methylation levels in the SH-SY5Y cells for both Act. D and Rosco ($P = 0.739$, one-way ANOVA, Fig. 2B), compared to the control.

3.4. COASY DNA methylation levels increase with expression of HSV-1 capsid protein VP26 in nucleus

We induced SH-SY5Y cells to express a fusion protein of VP26 and EGFP and a fusion protein of VP26, NLS and EGFP (Fig. 3A and B). Owing to the presence of NLS, VP26 was localized in the nucleus. COASY DNA methylation levels for these proteins and SH-SY5Y cells expressing EGFP only as a control were assumed to be normally distributed. SH-SY5Y cells that expressed VP26 in the nucleus had a level of DNA methylation around 15% higher compared to SH-SY5Y cells, for both with and without VP26 expression in the cytosol ($P = 0.020$, $P = 0.027$, respectively, one-way ANOVA with post hoc Tukey’s multiple comparisons test, Fig. 3C).

3.5. DNMT1, DNMT3A and DNMT3B mRNA levels post VP26 transfection

In order to clarify reasons for changes in COASY DNA methylation with nuclear VP26 expression, we measured the mRNA levels of DNMTs and ACTB. Individual DNMT mRNA levels were calculated as ratios to

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**Fig. 2.** COASY DNA methylation levels post IE protein expression
(A) Experimental design for expressing HSV-1 IE protein only. (B) DNA methylation levels in the COASY promoter region 24 h after expression of IE protein (control: $n = 6$, Act. D: $n = 8$, Rosco: $n = 8$). Columns with error bars indicate mean with SEM. ns: not significant.

**Fig. 3.** Capsid protein VP26 expression and COASY DNA methylation levels
We transfected VP26-EGFP fusion protein (A) and VP26-NLS-EGFP fusion protein (B) into SH-SY5Y cells and observed expression. Scale bar: 100 μm. (C) DNA methylation levels in COASY promoter region 24 h after transfection of fusion proteins (EGFP: $n = 6$, VP26: $n = 8$, VP26-NLS: $n = 9$). *$P < 0.05$ by one-way ANOVA with post hoc Tukey’s multiple comparisons test. ns: not significant.
We focused on the binding of the HSV-1 capsid protein VP26 with DNMT3A (Fig. 2B), which suggested that E or L proteins altered DNA methylation.

There was no significant change in DNA methylation levels suggested that DNA methylation levels decreased in HSV-1 lytic infection (Fig. 1B). The present study demonstrated for the first time that HSV-1 causes methylation of host DNA, indicating an association of AD and HSV-1 indirectly. It also showed that COASY methylation was induced by VP26, a HSV-1 capsid protein.

In this study, we aimed to identify the effects of HSV-1 infection on DNA methylation levels in the COASY promoter region. Our results suggested that DNA methylation levels decreased in HSV-1 lytic infection (Fig. 1A). On the other hand, when replication was inhibited by ACV, COASY DNA methylation levels increased (Fig. 1B). The present study demonstrated for the first time that HSV-1 causes methylation of host DNA, indicating an association of AD and HSV-1 indirectly. It also showed that COASY methylation was induced by VP26, a HSV-1 capsid protein.

Although the mechanism of HSV-1 reactivation has not been clarified, hyperthermia is known to be a reactivation triggering factor from mouse trigeminal ganglia (De Chiara et al., 2019; Sawtell and Thompson, 1992). Exposure to bright light, stress, and fatigue are triggers for the recurrence of cold sores in humans (Worrall, 2009). As HSV-1 reactivation is reportedly a risk for AD (Kobayashi et al., 2013; Proto et al., 2022), we considered it possible that expression of the capsid protein VP26 is associated with AD onset by modifying DNA methylation in the brain.

In lytic infection, as COASY DNA methylation decreased (Fig. 1A), and no significant change was observed for expression of IE protein alone (Fig. 2B), this suggests that E protein has relatively strong demethylation capability.

Since previous studies have demonstrated an interaction between VP26 and DNMT3A (Rowles et al., 2015), the increase in COASY DNA methylation levels was due to an increase in enzymatic activity or transcription of DNMTs. We found that there was a significant decrease in DNMT3A mRNA in SH-SY5Y cells with VP26 expression in the nucleus as compared to EGFP expression. In addition, there was a tendency for levels of DNMT3B mRNA in cells with VP26 expression in the nucleus to be lower, though the difference was not significant (Fig. 4C). Furthermore, while the reasons for these observations were not clear, our results suggested that VP26 binds with DNMT3A to reinforce DNA methylation capacity and as DNMT3A transcription undergoes negative feedback there is a decrease.

Epigenetic regulation of HSV-1 latent gene expression post-infection was previously reported (Bloom et al., 2010) but no study has yet demonstrated that altered DNA methylation in host cells is an epigenetic change due to HSV-1. This could also be related to the mechanism of

4. Discussion

In order to investigate whether changes in DNMTs at the transcriptional level are associated with changes in COASY DNA methylation, we quantified mRNA for DNMT1, DNMT3A and DNMT3B in cells expressing the various proteins mentioned in the foregoing, by real-time RT-PCR. The results showed that DNA methylation had not been triggered by an increase in the amount of DNMT expression.

The alteration of COASY DNA methylation due to the HSV-1 capsid protein VP26 is of great interest because an association between COASY methylation and AD has been observed (Kobayashi et al., 2016a, b; Kobayashi et al., 2020) and this could be indirect evidence for an association of HSV-1 infection and AD.

HSV-1 establishes latency in the trigeminal ganglia but details of a route by which it might enter the brain are unknown (Menendez and Carr, 2017). However, it has at least been shown experimentally that the virus is transported within axons as a capsid (Dong et al., 2020). This suggests that even if HSV-1 does not form virus particles in the brain, it is plausible that capsid protein is present and triggers DNA methylation in the brain.

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HSV-1 latent infection and reactivation.

Our study had a few limitations. First, we only identified the involvement of VP26 in DNA methylation. Other virus proteins could also exert an influence on DNA methylation. In particular, E protein may have a function that affects DNA demethylation. Thus, further investigation targeting the involvement of other viral proteins will be needed to better understand the process of DNA methylation during HSV-1 infection. Second, we focused specifically on the COASY gene. It is likely that there are other genes whose DNA methylation pattern is altered during HSV-1 infection and if such genes are found, they would be good candidates for further studies.

The present study demonstrated that COASY methylation, which is thought to be associated with AD, was caused by HSV-1 infection, in particular by the capsid protein VP26. This suggests that preventing infection by HSV-1, its reactivation and invasion into the brain might prevent AD. However, preventing HSV-1 infection through ACV therapy would not be sufficient. It would also be necessary to clarify the mechanism of HSV-1 reactivation and develop radical therapy to eliminate HSV-1 from the body.

In summary, we demonstrated that HSV-1 infection in the presence of ACV and expression of VP26, a capsid protein of HSV-1, resulted in interaction with DNMT3A, to achieve higher levels of DNA methylation in the COASY promoter region. Our results should help to identify a potential pathway by which HSV-1 infection might cause AD.

Funding
The present work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI (grant number 21K07553). The funders were not involved in the design of the study or conducting it; in the collection, management, analysis, and interpretation of the data; or in the preparation, review and approval of the manuscript.

Declaration of competing interest
The authors have no conflict of interest to report.

Data availability
Data will be made available on request.

Acknowledgments
We would like to thank Mr. Alexander Cox for editorial assistance with the manuscript.

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