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Epigallocatechin-3-gallate downregulates PDHA1 interfering the metabolic pathways in human herpesvirus 8 harboring primary effusion lymphoma cells

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ABSTRACT

Primary effusion lymphoma (PEL) is an aggressive neoplasm correlated with human herpesvirus 8 (HHV8). Metabolic reprogramming is a hallmark of cancers. The alterations in cellular metabolism are important to the survival of HHV8 latently infected cells. Pyruvate dehydrogenase (PDH) controls the flux of metabolites between glycolysis and the tricarboxylic acid cycle (TCA cycle) and is a key enzyme in cancer metabolic reprogramming. Glutaminolysis is required for the survival of PEL cells. Glutamate dehydrogenase 1 (GDH1) converts glutamate into α-ketoglutarate supplying the TCA cycle with intermediates to support anaplerosis. Previously we have observed that epigallocatechin-3-gallate (EGCG) can induce PEL cell death and *N*-acetyl cysteine (NAC) attenuates EGCG induced PEL cell death. In this study, results showed that EGCG upregulated the expression of glucose transporter GLUT3, and reduced the expression of pyruvate dehydrogenase E1-alpha (PDHA1), the major regulator of PDH, and GDH1. NAC could partially reverse the effects of EGCG in PEL cells. Overexpression of PDHA1 in PEL cells or supplement of α-ketoglutarate attenuated EGCG induced cell death. EGCG also reduced the levels of oncometabolite D-2-hydroxyglutarate (D2HG). These results suggest that EGCG may modulate the metabolism of PEL cells leading to cell death.

1. Introduction

Primary effusion lymphoma (PEL) is an aggressive tumor with poor prognosis and is associated with the human herpesvirus 8 (HHV8) (Nador et al., 1996). HHV8 is essential for the survival of PEL cells (Guasparri et al., 2004). Similar to the other herpesviruses, HHV8 can establish two distinct life cycles including lytic replication and latency (Ye et al., 2011). During lytic replication HHV8 modulates cellular metabolism to produce virus progenies and latent HHV8 infection alters metabolic pathways that are related to various cancer cells (Lagunoff, 2016).

Several core cellular metabolic pathways, including glycolysis, fatty acid synthesis and glutaminolysis, are adapted by viruses that is similar to the cancer hallmark, metabolic rewiring (Sanchez and Lagunoff, 2015). Aerobic glycolysis, also known as Warburg effect, can generate energy required to support rapid cell proliferation (Vander Heiden et al., 2009). Previous studies have demonstrated aerobic glycolysis is increased in PEL cells and inhibition of glycolysis can reduce the viability of PEL cells (Bhatt et al., 2012). Further evidence shows pyruvate kinase 2 (PKM2) is upregulated upon HHV8 infection in endothelial cells. The increase of PKM2 is HIF-dependent and is important for maintaining aerobic glycolysis in HHV8-infected cells (Ma et al., 2015). However, contradictory results reveal the expression of GLUT1 and GLUT3 is downregulated to restrict glucose uptake in HHV8-transformed cells resulting in the inhibition of aerobic glycolysis and oxidative phosphorylation (Zhu et al., 2016). Glutamine addict is the other metabolic signature observed in cancer cells. Aerobic glycolysis results in decrease of ATP production in cancer cells, but the demand for biosynthetic reactions is required to maintain cancer cell proliferation. Glutaminolysis is frequently elevated in cancer cells. Glutamine is transported into cells via SLC1A5, and is converted to glutamate, then to alpha-ketoglutarate (α-KG) to provide TCA intermediates

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(anapleurosis) and biosynthetic precursors for growing cancer cells, (Jin et al., 2016; Ratnikov et al., 2015). Increased glutamine uptake is observed in latent HHV8 infected cells. Depletion of glutamine causes apoptosis of HHV8 latently infected endothelial cells (Sanchez et al., 2015).

Epigallocatechin-3-gallate (EGCG), the major constituent of green tea, has been shown to contain cancer prevention and antitumor activities against various cancers (Fujiki et al., 2018). In vitro and in vivo studies suggest that the induction of ROS generation is responsible for the anti-cancer effect of EGCG (Min and Kwon, 2014). Previously we have demonstrated that EGCG induces ROS generation and cell death in HHV8 harboring PEL cells (Tsai et al., 2017). In the present study, we observed that EGCG treatment could disrupt the cellular metabolism of PEL cells. EGCG elevated the expression of GLUT3, downregulated the expression of pyruvate dehydrogenase E1 subunit (PDHA1) and glutamate dehydrogenase 1 (GDH1), and reduced the levels of D-2 hydroxyglutarate (D2HG). Overexpression of PDHA1 or supplement of α-KG could rescue the EGCG-induced PEL cell death. These data indicate that EGCG may interfere with the metabolism of HHV8 harboring PEL cells resulting cell death.

2. Materials and methods

2.1. Cell culture

BCBL-1 (ATCC CRL11982; a HHV8-positive and EBV-negative PEL cell line) cells, were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, and maintained at 37 °C under 5% CO_2.

2.2. Reagents and antibodies

EGCG (Sigma, St. Louis, MO, USA).was dissolved in H_2O at 5 mg/mL as a stock solution and added to culture medium at. 20 μg/mL. *N*-acetylcysteine (NAC) and Dimethylα-ketoglutarate (α-KG) (Sigma, St. Louis, MO, USA).were dissolved in PBS at 1 M and 50 mM as stock solution respectively. The primary antibodies against PDHA1 and GDH1 were obtained from Genetex (Irvine, CA). Anti- β-actin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibody against GLUT3 purchased from Abclonal, Inc.

2.3. Reverse transcription- polymerase chain reaction (RT-PCR) and RT-qPCR analysis

To analyze the expression levels of target genes, total RNA was extracted from BCBL-1 cells treated with EGCG (20 μg/mL) in the absence or presence of 10 mM NAC using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The purified mRNA samples were digested with DNase I (Promega) for 30 min at 37 °C, and the reaction was stopped by EDTA followed by heat inactivation at 70 °C. Reverse transcription-PCR (RT-PCR) was performed by using a SuperScript III (Invitrogen), and the synthesized cDNA samples were used as templates for PCR or qPCR using specific primers. Quantitative real-time PCR was performed using SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The following primer sequences were used: GLUT3 Forward, 5'-GGTGGAAAGGGCAGG.

AAGAA-3′ and reverse, 5′-AAATAGTGAGCAGCGGAGGG-3′; PDHA1 Forward, 5′-TCGCTATGGAATGGGAACGT-3′ and reverse, 5'-TCGCTGG AGTAGATGTGGTA-3′; GDH1 Forward, 5′-CATGGGTCCATTCTGGG CTT-3′ and reverse, 5′-CTGTCTTGGAA.

CTCTGCCGT-3′; β-actin Forward, 5′-CGTACCACTGGCATCGT GAT-3′ and reverse, 5′-CTTGATCTTCATTGTGCTGGGTG-3′. A relative quantity (RQ) value was used for the calculation of real-time PCR experiment. The $2^{-\Delta\Delta CT}$ method was adopted to analyze relative gene expression levels and β-actin gene was made as reference gene in all experiments.

2.4. Western blotting

Cell lysates were prepared using lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 0.25% sodium deoxycholate, 1 mM EDTA, 100 mM sodium chloride, 50 mM sodium fluoride, 5 mM sodium orthovanadate, 1× complete protease inhibitors (Roche)). Cell lysates were separated by SDS-PAGE gel electrophoresis and transferred to PVDF membranes (Millipore, MA). After blocking with 5% bovine serum albumin, membranes were incubated overnight at 4 °C with primary antibodies and corresponding HRP-conjugated secondary antibodies. The signals were detected using an enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) method.

2.5. Plasmid construction

The human PDHA1 cDNA sequence was amplified by PCR utilizing specific primers, forward, 5′-GAATTCTTCATGAGGAAGATGCTCGC-3′ and reverse, 5′-GGATCCTT.

AACTGACTGACTTAAACT-3′. Underlined letters indicate the restriction sites. The full length PDHA1 PCR product was inserted into the pEASY-T1 cloning vector to generate. pEASY-T1-PDHA1. PDHA1 expression plasmid, pIRES2-EGFP/PDHA1, was constructed by cloning PDHA1 obtained from *EcoR*I and *BamH*I digested pEASY-T1-PDHA1 into pIRES2-EGFP (Clontech, Palo Alto, CA).

2.6. Cell transfection and cell viability assay

pIRES2-EGFP or pIRES2-EGFP/PDHA1 was transfected into BCBL-1 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After 24 h transfection, the transfected PEL cells were suspended at 2×10^5 cells/ml and treated with EGCG (20 μg/mL) for 24 h. Cell viability was determined by trypan blue exclusion assay. The untreated cells were utilized as control (considered to be 100%), and the cell viability was compared with control. Each treatment was performed in triplicate and three independent experiments were performed. Error bars represent the standard errors.

2.7. Luciferase reporter assay

A luciferase reporter plasmid containing 5 copies of NF-κB response element and the.

pTK-RL (encoding Renilla luciferase) control plasmid were transfected into 293 cells. After 24 h posttransfection, the transfected cells were untreated or treated with EGCG (20 μg/mL), incubated for another 24 h and then luciferase activity was determined by the dual luciferase reporter assay system (Promega, Madison, WI, USA). The levels of reporter genes were normalized with renilla luciferase activity. The NF-κB luciferase activity in the untreated cells was used as control, relative luciferase activity was expressed as inhibition (%) relative to that of the untreated cells.

2.8. Enzymatic assay for D2HG

The levels of D2HG were analyzed by enzymatic assay for D-2HG (K213–100, BioVision, CA) according to the manufacturer's instructions. Briefly, PEL cells were untreated or treated with EGCG (20 μg/mL) for 24 h, and lysed in cold assay buffer provided by the manufacturer. The cell lysates were incubated with D-2-hydroxyglutarate dehydrogenase and D2HG substrate mix at 37 °C for 1 h, and then colometric assessment performed at OD450nm.

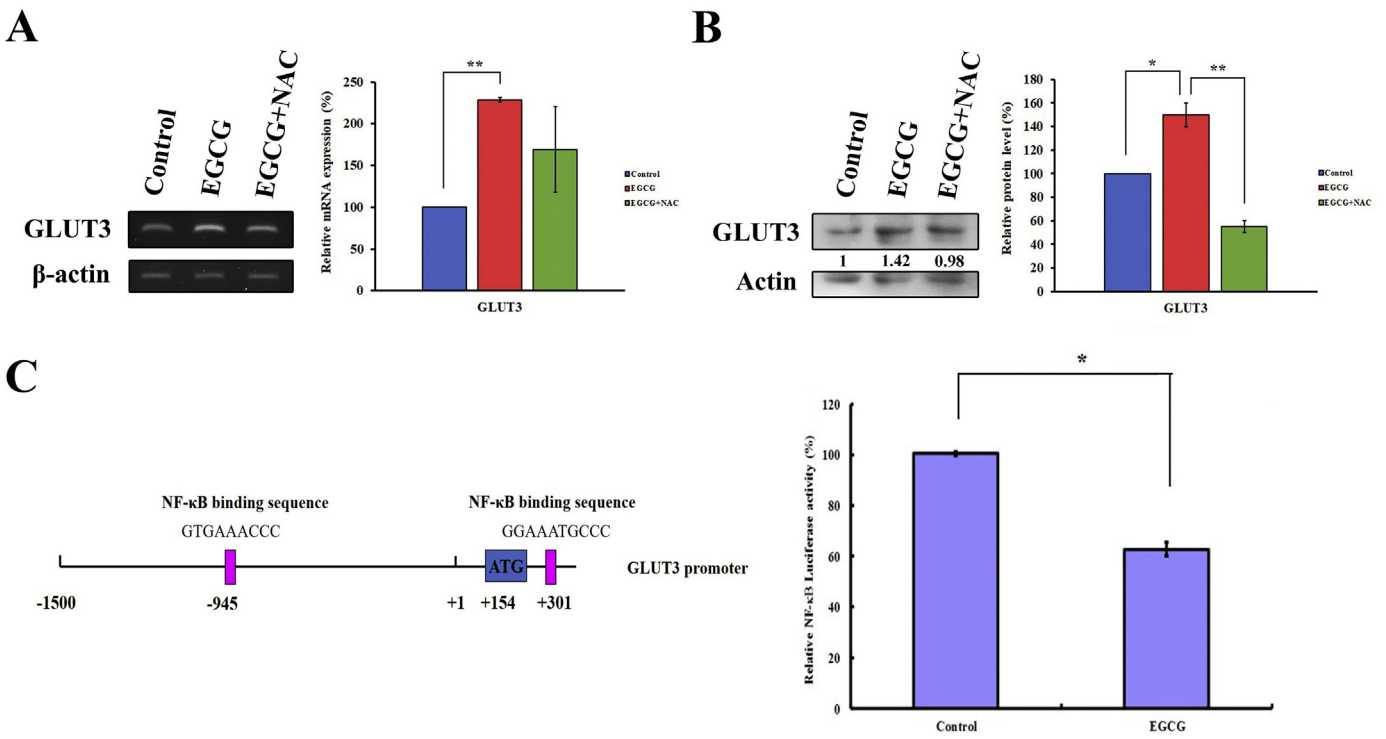

Fig. 1. EGCG increased the gene expression levels of GLUT3 in HHV8 harboring PEL cells. BCBL-1 cells were untreated or treated with 20 μg/mL EGCG for 24 h in the absence or presence of 10 mM *N*-acetyl-cysteine (NAC). (A) The mRNA levels of GLUT3 were determined by RT-PCR (left panel) and quantified by qRT-PCR (right panel). (B) The protein levels of GLUT3 were detected by western blot analysis. The representative data are shown in left panel, and the quantification data are shown in right panel. (C) The left panel showed the putative NF-κB binding sites identified in the GLUT3 promoter region. The right panel showed that EGCG suppressed the NF-κB driven luciferase activity. The relative gene expression was normalized to actin levels. The results are the means of three independent experiments, and bars represent the standard errors. $*p < .05$ and $**p < .01$, compared to controls.

2.9. Statistical analysis

Each experiment was performed at least three times. All data were presented as mean ± SEM and analysis by SPSS v20 using one-way ANOVA. $*p < .05$ and $**p < .01$ was considered statistically significant.

3. Results

3.1. EGCG induces GLUT3 expression in PEL cells

It has been reported that inhibition of aerobic glycolysis induces apoptosis in HHV8 infected endothelial cells (Delgado et al., 2010), but suppression of aerobic glycolysis has also been observed in HHV8 harboring PEL cells (Zhu et al., 2016). To observe the effect of EGCG on glycolysis, the expression of GLUT3 was determined in the EGCG treated PEL cells. Since NAC can attenuate EGCG induced cell death in PEL cells (Tsai et al., 2017), the expression of GLUT3 was also detected in the NAC and EGCG treated PEL cells. Results showed that EGCG elevated the expression of GLUT3 at both mRNA (Fig. 1A) and protein levels (Fig. 1B), and NAC could reduce the GLUT3 expression induced by EGCG (Fig. 1).

Previous studies exhibit NF-κB inhibitors can increase GLUT3 expression, indicating that NF-κB pathway may involve in the inhibition of GLUT3 expression in HHV8 transformed cells (Zhu et al., 2016). As shown in Fig. 1C, two putative NF-κB binding sites were identified in the promoter region of GLUT3. To test whether EGCG could affect NF-κB activation, a luciferase reporter plasmid containing 5 copies of NF-κB response element was transfected into 293 cells, results revealed that EGCG decreased the activation of NF-κB (Fig. 1C). These results suggest that EGCG might suppress NF-κB activation to enhance the expression of GLUT3.

3.2. EGCG suppresses PDHA1 and GDH1 expression in PEL cells

Pyruvate dehydrogenase E1 subunit (PDHA1) is the major regulator for the metabolic switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis. PDHA1 can catalyze the oxidative decarboxylation of pyruvate to acetyl-CoA for TCA cycle. Alternatively, pyruvate can be converted to lactic acid by Lactate Dehydrogenase A (LDHA) (McFate et al., 2008; Patel et al., 2012). Since EGCG treatment increased the expression of GLUT3 in PEL cells, the effect of EGCG on PDHA1 was further examined. Results revealed that EGCG treatment inhibited the expression of PDHA1 at both mRNA (Fig. 2A) and protein levels (Fig. 2B). NAC could attenuate the EGCG induced decrease of PDHA1 expression (Fig. 2A & B).

It has been reported that HHV8 promotes glutamine metabolism by increasing the expression of various enzymes, including glutamate dehydrogenase 1 (GDH1) for cell proliferation (Zhu et al., 2017). GDH is upregulated in various cancers. EGCG has been shown to inhibit GDH modulating insulin secretion (Li et al., 2006). Therefore, the expression of GDH1 was detected upon EGCG treatment. As expected, the expression of GDH1 was reduced in EGCG treated PEL cells and NAC could rescue the downregulation of GDH1 induced by EGCG (Fig. 2C & D).

3.3. EGCG treatment decreases the levels of D2HG in PEL cells

D-2 hydroxyglutarate (D2HG), an oncometabolite, is normally present at low levels in human cells, but cellular D2HG levels are elevated in IDH-mutated tumor cells (Achouri et al., 2004; Dang et al., 2009; Gross et al., 2010). High levels of D2HG block the TET family of DNA demethylases and Jumonji family of histone demethylases leading to epigenetic changes and altering gene expression (Xu et al., 2011). Increased glutamine uptake and glutaminolysis can cause 2-HG

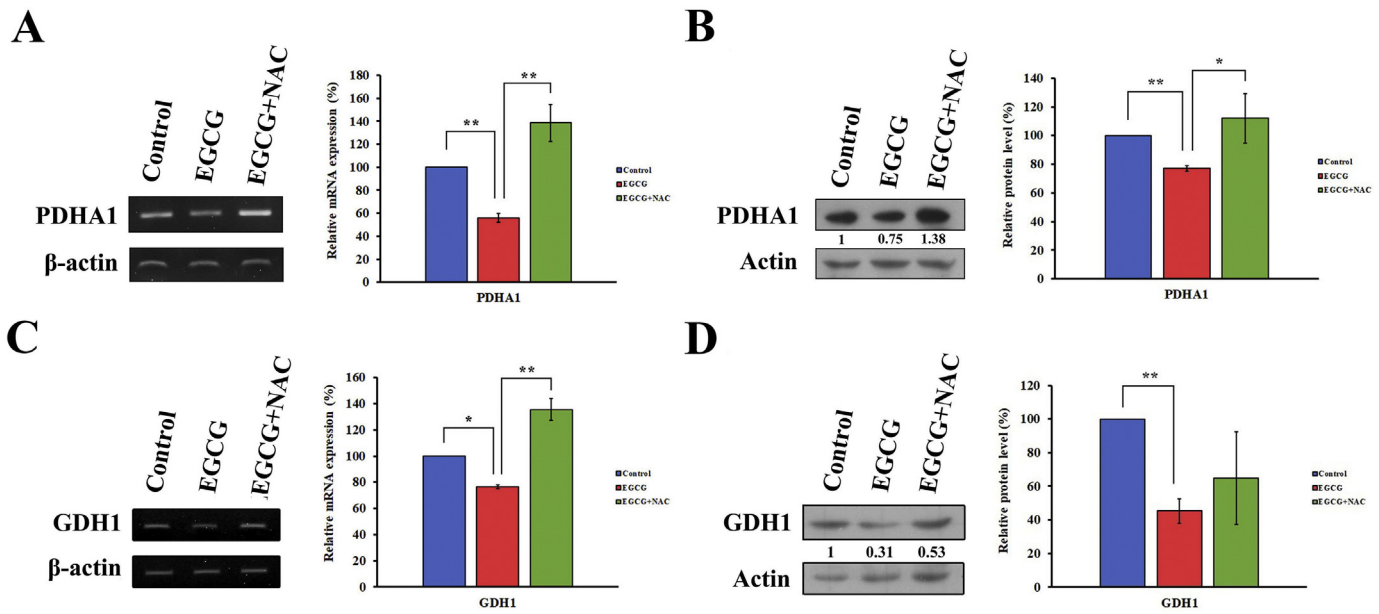

Fig. 2. EGCG downregulated the gene expression levels of PDHA1 and GDH1 in HHV8 harboring PEL cells. BCBL-1 cells were untreated or treated with 20 μg/mL EGCG for 24 h in the absence or presence of 10 mM *N*-acetyl-cysteine (NAC). (A) The mRNA levels of PDHA1 were determined by RT-PCR (left panel) and quantified by qRT-PCR (right panel). (B) The protein levels of PDHA1 were detected by western blot analysis. The representative data are shown in left panel, and the quantification data are shown in right panel. (C) The mRNA levels of GDH1 were determined by RT-PCR (left panel) and quantified by qRT-PCR (right panel). (D) The protein levels of GDH1 were detected by western blot analysis. The representative data are shown in left panel, and the quantification data are shown in right panel. The relative gene expression was normalized to actin levels. The results are the means of three independent experiments, and bars represent the standard errors. *p < .05 and **p < .01, compared to controls.

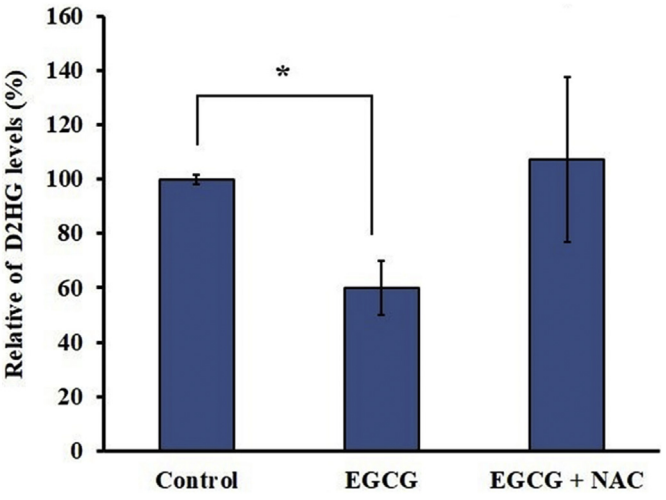

Fig. 3. EGCG decreased the oncometabolite D2HG levels in HHV8 harboring PEL cells. BCBL-1 cells were untreated or treated with 20 μg/mL EGCG for 24 h. The D2HG levels was determined by D-2-Hydroxyglutarate assay kit. The results are the means of three separate experiments, and bars represent the standard deviation. The results are the means of three independent experiments, and bars represent the standard errors. *p < .05, compared to controls.

accumulation lacking IDH mutation in breast cancer cells (Smolkova et al., 2015). Even though there was no IDH mutation observed in PEL cell lines (unpublished data), HHV8 infected cells become glutamine addicts has been reported (Sanchez et al., 2015). Since EGCG treatment inhibited GDH1 expression interfering glutaminolysis, the levels of D2HG was measured in EGCG treated PEL cells. As shown in Fig. 3, compared with the untreated cells, the levels of D2HG in PEL cells were reduced about 50% upon EGCG treatment.

3.4. Supplement of α-KG rescued EGCG induced PEL cell death

In glutaminolysis, glutamine is converted to glutamate by glutaminase and then glutamate is catalyzed by GDH1 to produce α-KG. α-KG, a TCA cycle intermediate, can enter the mitochondria to replenish the TCA cycle where it provides intermediates for other biosynthetic reaction and supports energy production (Jin et al., 2016). Since EGCG inhibited the expression of both PDHA1 and GDH1, the source of α-KG was blocked that may cause PEL cell death. Therefore, the methyl α-KG was supplemented into culture medium and the viability of EGCG treated PEL cells was evaluated. The results showed that supplement of α-KG could rescue EGCG induced PEL cell death (Fig. 4A).

3.5. Overexpression of PDHA1 attenuated EGCG induced PEL cell death

To elucidate whether downregulation of PDHA1 is critical for the EGCG induced PEL cells, we constructed a PDHA1 overexpressing plasmid, pIRES2-EGFP/PDHA1. PEL cells were transfected with pIRES2-EGFP/PDHA1 or the control vector; 24 h later, cells were treated with EGCG and the viable cells determined by trypan blue staining after a further 24-h period. Fig. 4B showed overexpression of PDHA1 in PEL cells attenuated EGCG-induced cell death. These results indicate that overexpression of PDHA1 partially enhances the resistance of PEL cells to the EGCG.

4. Discussion

Cancer cells exhibit unusual metabolic activities to satisfy their bioenergetic and biosynthetic demand. Interruption of cancer metabolism can be a strategy for cancer therapy. The aim of this study was to explore the effects of EGCG on cancer cell metabolism. Using HHV8 latently infected PEL cells we showed that EGCG inhibited the cancer metabolism, including glycolysis and glutaminolysis. EGCG treatment also decreased the levels of oncometabolite D2HG. The possible mechanism is summarized in Fig. 5.

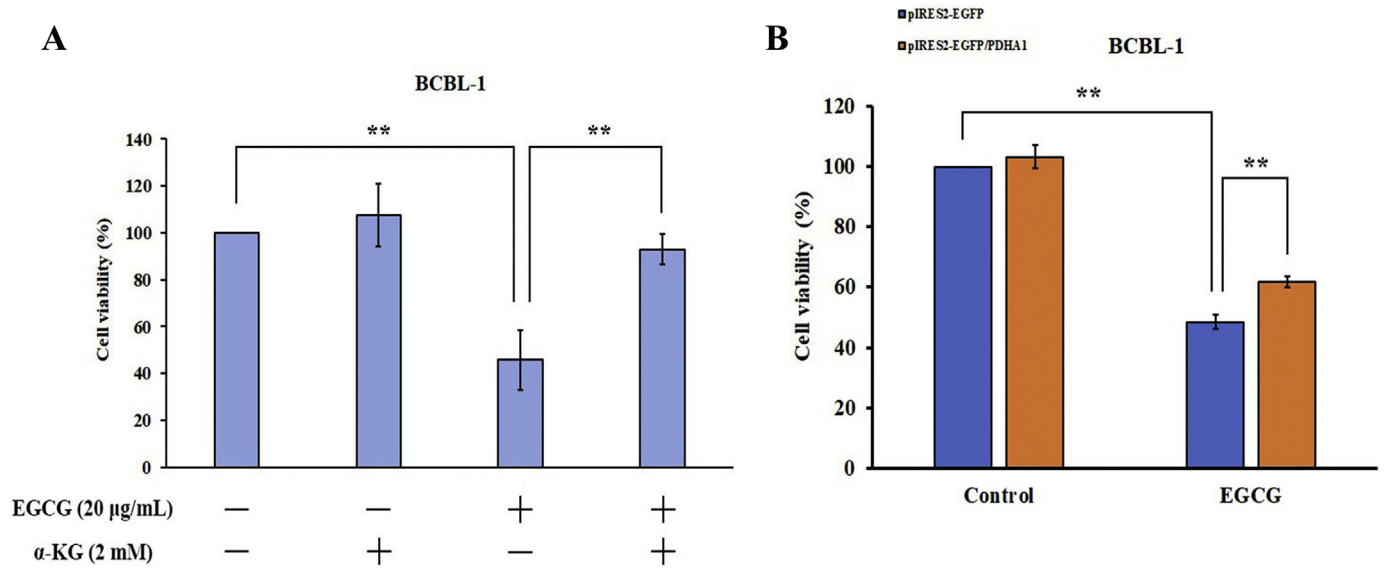

Fig. 4. Compensation of TCA cycle by Supplement with α-ketoglutarate or overexpressing PDHA1 rescued EGCG-induced PEL cell death. (A) Supplement of α-KG rescued EGCG-induced PEL cell death. BCBL-1 cells were untreated or treated with 20 μg/mL EGCG for 24 h in the absence or presence of 2 mM α-ketoglutarate. (B) Overexpression of PDHA1 attenuated EGCG-induced PEL cell death. BCBL-1 cells were transiently transfected with pIRES2 or pIRES/PDHA1. At 24 h post-transfection, the transfected BCBL-1 cells were untreated or treated with 20 μg/mL EGCG for 24 h. Cell viability was determined by trypan blue exclusion assay. The results are the means of three separate experiments, and bars represent the standard deviation. **$p < .01$, compared to controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Recent studies suggest that suppression of Warburg effect in tumor cells is unable to inhibit tumor growth because tumor cells can switch their metabolism to oxidative phosphorylation (de Padua et al., 2017; Zdralevic et al., 2018). EGCG could decrease the expression of PDHA1 (Fig. 2A) and also inhibited the expression of lactate dehydrogenase A (LDHA) (Supplementary Fig. 1C) in PEL cells, indicating that EGCG induced PEL cell death may due to suppresses both the aerobic glycolysis and oxidative phosphorylation.

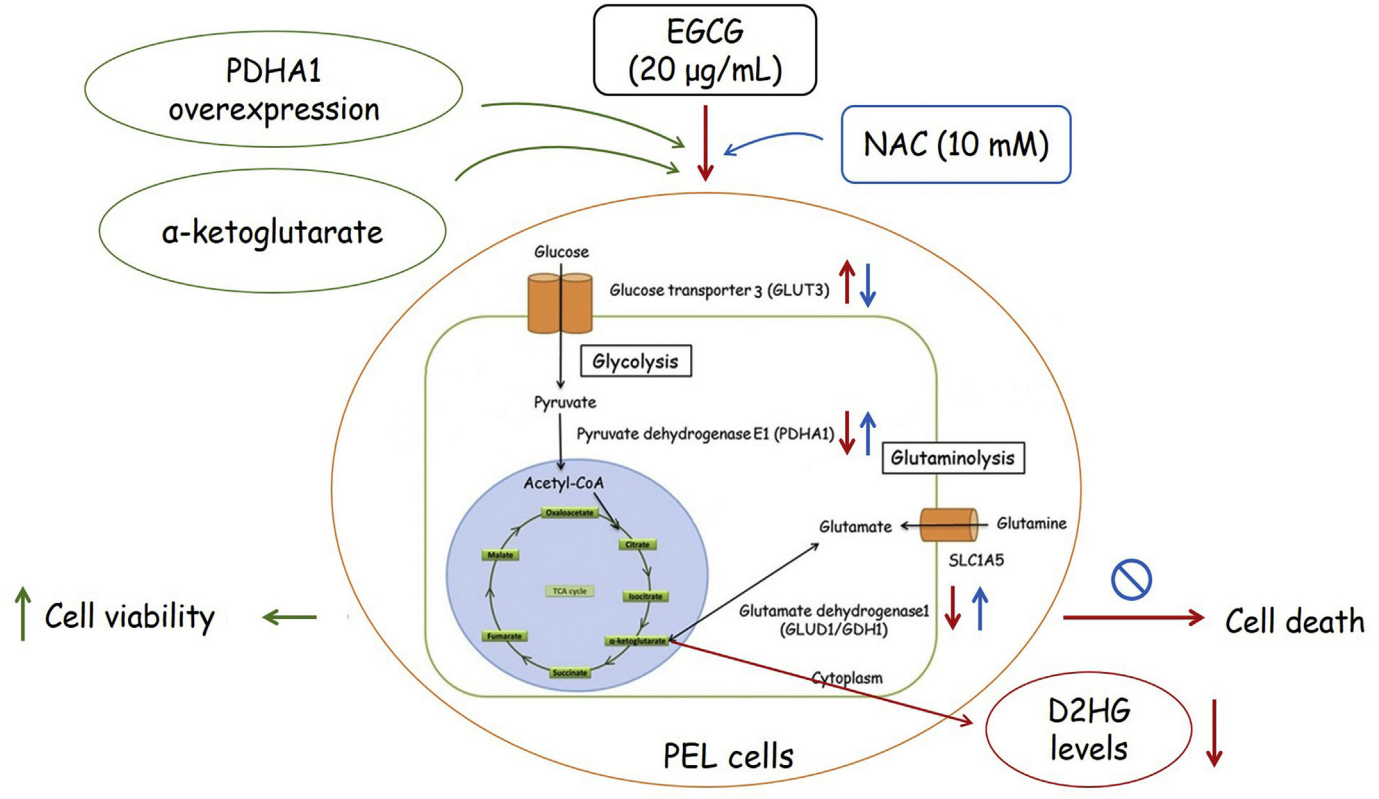

Fig. 5. A proposed mechanism of EGCG disrupts the metabolic pathways of PEL cells. The red arrows indicate that EGCG interferes in glycolysis and glutaminolysis in PEL cells by upregulating GLUT3 and downregulating PDHA1 and GDH1. EGCG also reduces D2HG levels. The blue arrows indicate that NAC can partially reverse the effects of EGCG. The green arrows indicate that supplement of α-KG or overexpressing of PDHA can rescue EGCG-induced PEL cell death. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TCA cycle is pivotal for macromolecule synthesis, energy metabolism and redox balance. Accumulating evidence indicates cancer cells become more dependent on TCA cycle for macromolecule synthesis (Anderson et al., 2018). Due to aerobic glycolysis, cancer cells turn into glutamine addict and increase of glutaminolysis to replenish TCA cycle intermediates. Pyruvate dehydrogenase (PDH) is a multienzyme complex that converts pyruvate to acetyl-CoA by irreversible decarboxylation. PDHA1 is the major component of PDH, decrease of PDHA1 reduces pyruvate entering into TCA cycle (Rajagopalan et al., 2015). Our results revealed that EGCG inhibited both PDHA1 and GDH1, and overexpressing of PDHA1 could attenuated EGCG induced PEL cell death. These data indicate that EGCG downregulates PDHA1 and GDH1 expression to block the TCA cycle resulting PEL cell death. We also found that EGCG treatment could increase the expression of glucose transporters, GLUT1 and GLUT3 (supplementary Fig. 1A and Fig. 1), and the glutamine transporter, SLC1A5 (supplementary Fig. 1B). It has been reported that exposure of sertoli cells to 50 μM EGCG inhibited the proliferation and increased the glucose consumption of sertoli cells (Dias et al., 2017). PEL cells may compensate for the reduction of glucose and glutamine feeding the TCA cycle induced by EGCG through the increase of the expression of glucose transporters and glutamine transporter. Alternatively, EGCG just directly inhibited NF-κB activation to increase GLUT3 expression.

α-KG is an antioxidant. It can scavenge oxidative stress and has been widely applied in human for trauma, postoperative recovery, cancer and age-related diseases (Liu et al., 2018). Our results showed that supplement of α-KG could rescue EGCG induced cell death in PEL cells. The other antioxidant, NAC could attenuate the decrease of PDHA1 and GDH1 expression induced by EGCG (Fig. 2), it also decreased the EGCG induced PEL cell death (Tsai et al., 2017). These data suggest that EGCG might alter PEL cell metabolism via ROS generation. Recent studies demonstrate that taking antioxidant supplements increases tumor growth. It has been suggested that patients should avoid intake of antioxidant supplements during chemotherapy or radiation therapy (Jung et al., 2019). Similar to the other antioxidants, α-KG may be not a suitable supplement for cancer patients.

Mutations in IDH1 and IDH2 are observed in various cancer types, including gliomas and leukemias. IDH mutations cause the increase of production and accumulation of the oncometabolite D2HG (Ward et al., 2010). Previous studies have demonstrated that the elevated of D2HG in the IDH1/2 wild type cells is due to glutamine anaplerosis (Colvin et al., 2016), Glutamine anaplerosis is also observed in HHV8 infected cells (Sanchez et al., 2015). In this study, we found that EGCG reduced the levels of D2HG in HHV8 harboring PEL cells (Fig. 3). Since EGCG inhibited the expression of PDHA1 and GDH1 to block the TCA cycle (Fig. 2), EGCG may decrease the TCA cycle intermediates leading to the reduction of D2HG.

PEL is HHV8 harboring cells. HHV8 infection can alter cellular metabolism similar to the cancer hallmark, metabolic reprogramming. Previous studies have demonstrated that HHV8 microRNAs and vFLIP can downregulate GLUT1 and GLUT3 to inhibit glycolysis (Zhu et al., 2016). We have observed that EGCG treatment interfered PEL cell metabolism. Further experiments are necessary to explore whether viral genes are involved in EGCG disrupting PEL cell metabolism.

In conclusion, we observed that GLUT3 expression was upregulated in EGCG treated PEL cells. EGCG treatment downregulated PDHA and GDH1 expression, and decreased the levels of oncometabolite D2HG. Overexpressing of PDHA1 or supplement with α-KG could rescue EGCG induced PEL cell death. These results suggest EGCG could be a potential therapeutic agent for PEL treatment and also improved understanding of molecular mechanism of EGCG in cancer therapy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2019.104753.

Declaration of Competing Interest

The authors declare no conflict of interest.

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