

Dihydroartemisinin suppresses the proliferation of Epstein-Barr virus-associated gastric carcinoma cells via downregulation of latent membrane protein 2A

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Abstract. Treatment of recurrent and metastatic Epstein-Barr virus-associated gastric carcinoma (EBVaGC) remains a challenge, particularly in developing countries, due to lack of efficient screening programs. Latent membrane protein 2A (*LMP2A*) has been reported to serve an important function in the development of EBVaGC. In previous years dihydroartemisinin (DHA), traditionally used as an anti-malarial agent, has been demonstrated to inhibit tumor growth with low toxicity to normal cells. In the present study, the anti-tumor effect of DHA in EBVaGC was investigated. The MTT assay was used to compare the viability of untreated and DHA-treated EBVaGC GT-38 cells. Flow cytometry was applied to determine the percentage of GT-38 cells at each stage of the cell cycle. Reverse transcription-polymerase chain reaction and western blotting were used to determine the expression of the *LMP2A* gene. The effect of DHA treatment *in vivo* was evaluated in nude mice bearing GT-38 tumors. The results of the present study revealed that DHA-treated cells exhibited a time- and dose-dependent inhibition of viability. DHA significantly increased the apoptotic rate of GT-38 cells following treatment with 20 μ g/ml DHA for 48 h. DHA-treated GT-38 cells were blocked in the G₀/G₁ phase, resulting in an accumulation of G₀/G₁ phase cells and a significant decrease

of G₂/M phase cells. *In vivo*, the results of the present study revealed that DHA significantly inhibited the growth of GT-38 cell-transplanted tumors. The mRNA and protein levels of *LMP2A* were significantly downregulated in the DHA-treated group compared with the control group. The present data indicated that DHA inhibited cell growth and induced cell apoptosis of the EBVaGC GT-38 cell line via downregulation of *LMP2A*. DHA may therefore be a potential therapeutic candidate for the treatment of EBVaGC.

Introduction

Epstein-Barr virus (EBV), a human γ herpes virus, may exist in humans for a long time without producing any symptoms (1). A variety of human malignancies, including Burkitt's lymphoma (2), nasopharyngeal carcinoma (3) and gastric cancer have been reported to be associated with EBV (4). EBV-associated gastric carcinoma (EBVaGC) has been reported to account for $\leq 10\%$ of total gastric carcinoma (5). It is difficult to remove and eliminate EBVaGC cells thoroughly by surgical, radio and chemotherapeutic methods. Therefore, it is necessary to identify novel therapeutic approaches to treat EBVaGC by inhibiting tumor cell growth or survival.

At present, traditional Chinese herbs are widely being studied to treat numerous diseases (6,7). One herb, artemisinin, the active constituent of *Artemisia annua* L., along with its derivatives, has been used as an effective anti-malarial agent (8). Dihydroartemisinin (DHA), one of the main active metabolites of artemisinin, has been reported to exhibit anti-tumor activity in various cancer cells *in vitro* and *in vivo* in mice (9,10). DHA inhibits cell proliferation by inducing G₁ arrest and apoptosis in human nasopharyngeal carcinoma cells. DHA, as a putative signal transducer and activator of transcription 3 (STAT3) inhibitor, suppresses the growth of head and neck squamous cell carcinoma by targeting Janus kinase 2/STAT3 signaling. DHA prevents breast cancer-induced osteolysis by inhibiting breast cancer cells and osteoclasts. DHA combined with holotransferrin, which increases the concentration of ferrous iron in cancer cells, was reported to effectively kill a type of radiation-resistant human

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breast cancer cell *in vitro* (11). However, there are few studies about the effect of DHA on EBVaGC.

EBVaGC expresses a well-defined set of latent viral genes, including latent membrane protein 2A (*LMP2A*). It was reported that *LMP2A* is expressed in ~50% of EBVaGCs (12). As a transmembrane protein, it functions in multiple signal transduction pathways and is involved in the tumorigenic processes in EBVaGC (12). *LMP2A* is associated with DNA methyltransferases and induces expression of phosphorylated-STAT3, which causes upregulation of DNA methyltransferase (DNMT)1 (13) and DNMT3B (14) in EBVaGC cells. Downregulating *LMP2A* may also inhibit apoptosis through upregulation of the cellular survivin gene via the nuclear factor- κ B pathway (15). Therefore, *LMP2A* may be a potential target for treatment of EBVaGC.

In the present study, the effect of DHA on the growth of EBVaGC cells was explored and the *LMP2A*-associated mechanisms were investigated, with the aim of finding a potential candidate for EBVaGC therapy.

Materials and methods

Cell line and culture. The EBVaGC GT-38 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum (MP Biomedicals, LLC, Santa Ana, CA, USA), 100 U/ml penicillin and 100 mg/l streptomycin in a humidified environment with 5% CO₂.

MTT assay. GT-38 cells were seeded onto 96-well plates at 6x10⁴/ml. No DHA was added to the negative control group; equal amounts of PBS were added instead, and 5, 10, 20 and 40 μ g/ml DHA were added to the experimental groups of GT-38 cells for 48 h. Each group was repeated six times. A total of 20 μ l MTT solution (5 mg/ml) was added to each well following 0, 12, 24, 36, 48 and 60 h of incubation at 37°C, respectively, and the plates were incubated for 4 h at 37°C. Dimethyl sulfoxide (DMSO; 100 μ l) was then added to each well, and the plates were rotated for 15 min. The absorbance was measured at 450 nm with a microplate reader (SPECTRA MAX 190; Molecular Devices, LLC, Sunnyvale, CA, USA). The inhibition of cancer cell viability was calculated as follows: Inhibition rate (%)=(1-optical density (OD)_{treatment}/OD_{control}) x100.

Apoptosis assay. According to protocol of the Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), following treatment with 0 and 20 μ g/ml DHA for 48 h, GT-38 cells were harvested by centrifugation at 500 x g for 5 min at room temperature, resuspended in binding buffer and successively incubated with 5 μ l Annexin V-fluorescein isothiocyanate and 5 μ l propidium iodide (PI) for 15 min at room temperature. A flow cytometer (FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA) was then used to analyze the apoptosis results.

Analysis of cell cycle by flow cytometry. Following pre-incubation with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 24 h, 0 and 20 μ g/ml DHA was added to GT-38 cells for

48 h. The cells were centrifuged at 500 x g for 5 min at room temperature, fixed in 70% cooled ethanol, and then dyed with PI for 30 min in the dark. The cells were measured by a flow cytometer (FACS Calibur; BD Biosciences), and the data on the percentage of cells at each stage of the cell cycle was harvested.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from groups of GT-38 cells, which were treated with 0 and 20 μ g/ml DHA for 48 h, using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was then treated with DNaseI (Roche Diagnostics, Basel, Switzerland) to remove contaminating genomic DNA prior to the preparation of cDNA. Reverse transcription was performed using the cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). The following reagents were added into a sterile, nuclease free tube on ice in the following order: 1 μ l total RNA, 1 μ l Oligo (dT) 18 primer, 10 μ l nuclease-free water, 4 μ l 5X Reaction Buffer, 1 μ l RiboLock RNase Inhibitor (20 U/ μ l), 2 μ l 10 mM dNTP Mix and 1 μ l RevertAid M-MuLV RT (200 U/ μ l). The total volume was 20 μ l. The mix was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min. cDNA (5 μ l) from each sample was used to perform PCR. The sequences of primers for *LAMP2A* were as follows (13), forward: 5'-ATGACTCATCTCAACACATA-3' (nt.166874-166893), reverse: 5-CATGTTAGGCAAATTGCAA-3 (nt.166380-166361). The product size of *LMP2A* was 280 bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control, and the primers of GAPDH were as follows, forward: 5'-ACGGATTTGGTCGTATTGGG-3' and reverse: 5'-TGATTTTGGAGGGATCTCGC-3'. The qPCR was performed with 1 μ l cDNA using SYBR Green Taq Mix (Takara Bio, Inc., Otsu, Japan) on ABI PRISM 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression of target gene was evaluated using a relative quantification method (2^{- $\Delta\Delta C_q$}) (16), with GAPDH as the internal reference. The thermocycling conditions were as follow: 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, with a final extension step of 72°C for 5 min.

Western blot analysis. Protein was extracted from GT-38 cells treated with 0 or 20 μ g/ml DHA for 48 h using radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% nonidet P-40 and 50 mM Tris pH 8.0) with the addition of 2 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined with a The QuantiPro bicinchoninic acid (BCA) Assay kit (Sigma-Aldrich; Merck KGaA), and 20 μ g protein was loaded on 15% SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes for western blotting. Membranes were blocked at 4°C overnight with 5% skim milk, and incubated with a 1:200 dilution of rat anti-EBV *LMP2A* antibody at 4°C overnight (cat. no. ab59028; Abcam, Cambridge, UK). The blots were washed with phosphate buffered saline (PBS) with 0.05% Tween-20 3 times and then incubated with a donkey anti-rat secondary antibody conjugated with horseradish peroxidase, diluted by 1:2,000 (cat. no. A18739; Thermo Fisher Scientific, Inc.).

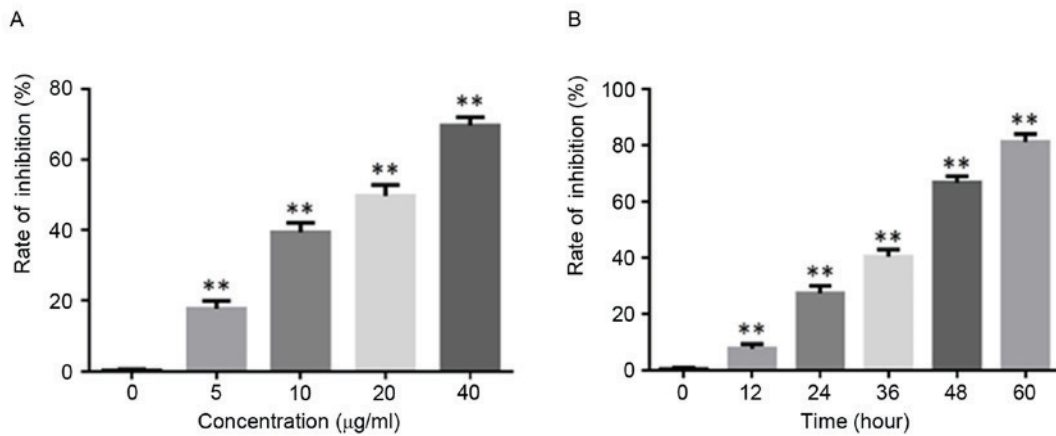


Figure 1. Effect of DHA on GT-38 cell viability. (A) Association between the rate of inhibition and concentration of DHA. (B) Association between the rate of inhibition and duration of DHA treatment. ** $P < 0.01$ vs. control ($n = 3$). DHA, dihydroartemisinin.

In vivo tumor growth xenograft models. A total of 12 BALB/c-nude mice (4-6-week-old; male; SPF degree, and 20-24 g of body weight) were purchased from the Sino-British Sippr/BK Lab Animal Co., Ltd., (Shanghai, China). Animals were housed in a specific pathogen-free room within the animal facilities, and were maintained at the average temperature of 23°C and the relative humidity of 40-70% in a 12-h light/dark cycle. The access to the food was restricted. All animals were allowed to acclimate to their new environment for 1 week prior to the initiation of the model. Mice were randomly divided into 2 groups (6 mice/group). The animal use protocol was approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University Second Affiliated Hospital (Xi'an, China). The GT-38 xenograft tumor model was developed by injecting a 5×10^6 GT-38 cell suspension into the right flank of the BALB/c-nude mice. Tumor nodules were allowed to grow to a volume of $\sim 150 \text{ mm}^3$ prior to initiating treatment. Tumor-bearing BALB/c-nude mice were randomly assigned to two groups and treated with 25 mg/kg DHA or DMSO. The tumor volume and total body weight were measured every 2 days. The tumor volume was calculated using the following equation: Tumor volume (V) = length \times width \times width $\times 0.52$. These experiments were replicated 3 times.

Statistical analysis. The continuous variables are presented as the mean \pm standard deviation. The statistical analysis was performed using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) followed by a Student Newman-Keuls post-hoc test. As treatment and time course were investigated, two-way ANOVA followed by a Student Newman-Keuls post-hoc test was also used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DHA inhibits GT-38 cell viability. The MTT assay revealed that DHA inhibited the viability of GT-38 cells in a dose-dependent manner (Fig. 1A). With increasing concentrations of DHA, the absorbance at 450 nm decreased and the rate of inhibition

increased ($P < 0.05$; $n = 6$; Fig. 1A). It was also observed that DHA exerted a time-dependent inhibition of viability in GT-38 cells (Fig. 1B). Following treatment with DHA for 0, 12, 24, 36, 48 and 60 h, the absorbance at 450 nm decreased and the rate of inhibition increased ($P < 0.05$; $n = 6$; Fig. 1B). Since there was no significant difference in cell growth inhibition between 48 and 60 h, 48 h was selected as the DHA treatment time for the subsequent experiments.

DHA treatment induces apoptosis and alters the cell cycle profile in GT-38 cells. Flow cytometric analysis revealed that the apoptotic rate was $6.25 \pm 0.38\%$ and $17.33 \pm 0.79\%$ following treatment with 0 and 20 $\mu\text{g/ml}$ DHA for 48 h, respectively (Fig. 2). The proportion of apoptosis was significantly increased in the 20 $\mu\text{g/ml}$ DHA group compared with the control group ($P < 0.05$; $n = 6$; Fig. 2).

It was revealed that the proportion of G_0/G_1 phase cells was $19.15 \pm 1.28\%$ following treatment with 20 $\mu\text{g/ml}$ DHA for 48 h, while the proportion of G_0/G_1 phase cells was $6.75 \pm 0.37\%$ in the control group. The present results also demonstrated that the percentage of G_2/M phase cells was $18.33 \pm 0.49\%$ subsequent to being treated with 20 $\mu\text{g/ml}$ DHA for 48 h, while the percentage of G_2/M phase cells was $31.15 \pm 1.78\%$ in the control group (Fig. 2A and B). The frequency of GT-38 cells in the G_0/G_1 phase was significantly increased while the frequency in the G_2/M was decreased following treatment with 20 $\mu\text{g/ml}$ DHA, as compared with the control group ($P < 0.05$; $n = 6$; Fig. 2C and D). No significant difference was observed in the S-phase phase between the control group and the DHA treated group.

DHA treatment inhibits the growth of GT-38 cell-transplanted tumors in vivo. Subcutaneous tumors were observed in all nude mice ($n = 12$) inoculated with GT-38 cells (tumorigenicity rate of 100%). Tumor-bearing mice treated with 25 mg/kg DHA exhibited a significant reduction in tumor volume compared with untreated mice. Tumor volumes were $1195.40 \pm 21.3 \text{ mm}^3$ and $220.98 \pm 12.5 \text{ mm}^3$ in untreated and DHA-treated mice bearing GT-38 tumors, respectively ($P < 0.01$; $n = 6$; Fig. 3). No significant changes of mice body weight between the control and DHA treated group were observed, which indicated that DHA treatment *in vivo* had no significant toxicity (Fig. 3).

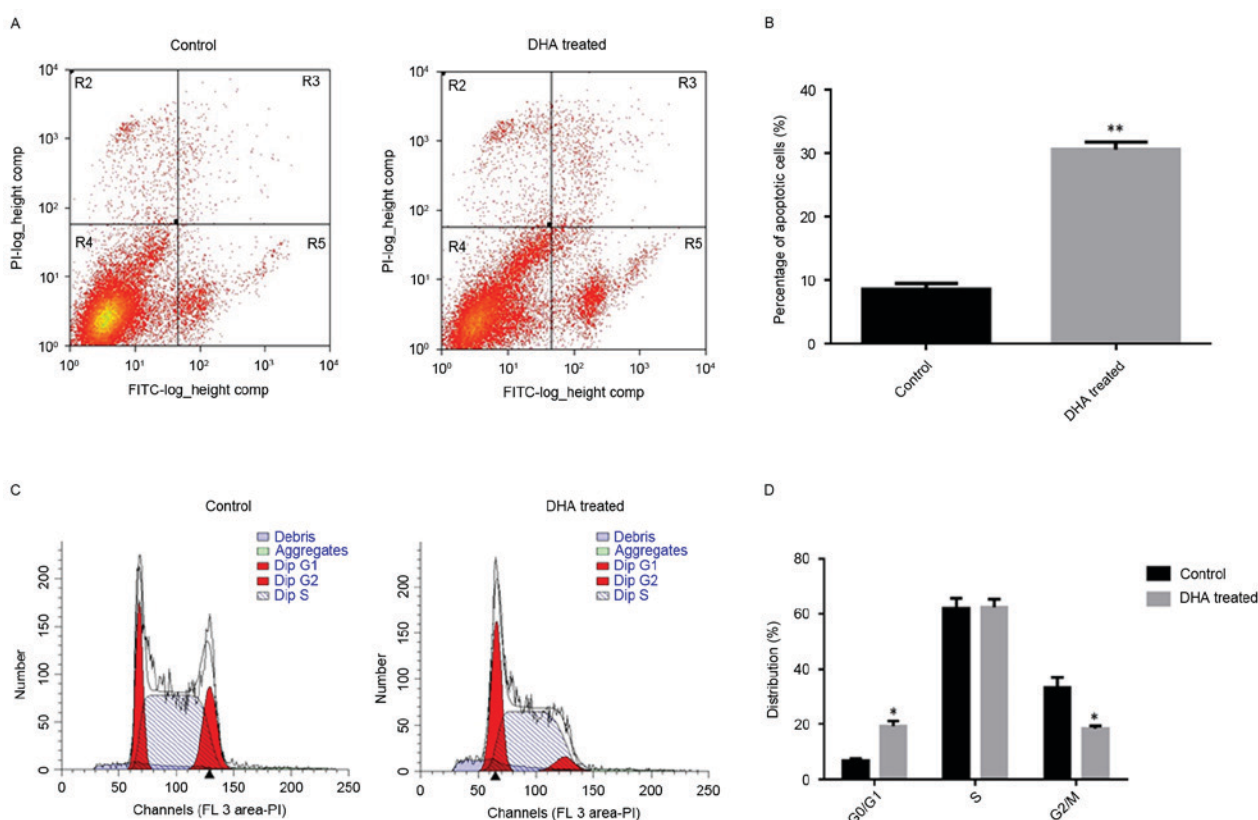


Figure 2. DHA treatment induced apoptosis and altered the cell cycle distribution in GT-38 cells (A) FCM revealed the apoptotic rate of GT-38 cells in the control and DHA-treated groups. (B) DHA significantly promoted cell apoptosis in the treated group. (C) FCM revealed each phase of GT38 cells in the cell cycle. (D) The proportion of cells in the G₀/G₁ phase significantly increased while the proportion in the S phase decreased in the DHA-treated group. * $P < 0.05$, ** $P < 0.01$ vs. control group (n=3). DHA, dihydroartemisinin; FCM, flow cytometry.

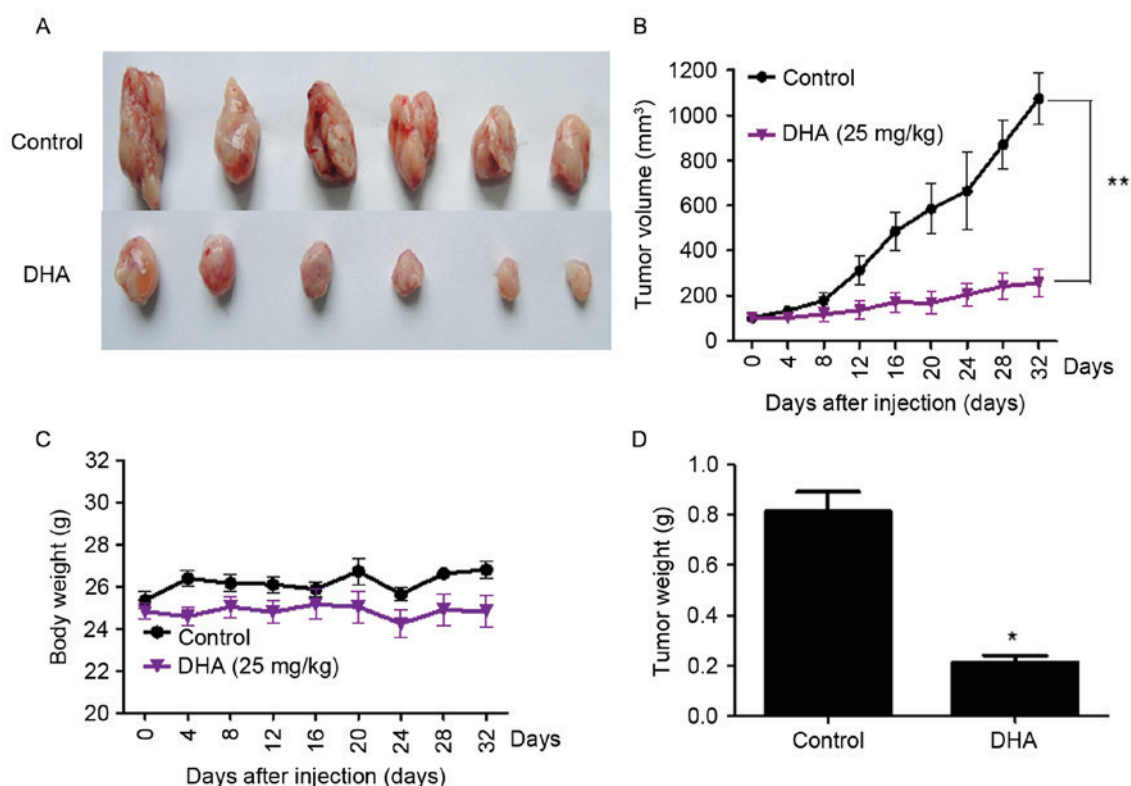


Figure 3. Antitumor effects of DHA *in vivo* and toxicity evaluation. (A) GT-38 tumor-bearing nude mice were treated with 25 mg/kg DHA or DMSO. Images of tumors in the two different groups at 32 days. (B) Treatment with DHA resulted in significant inhibition of tumor volume compared with the DMSO control from day 8. (C) No significant changes of body weight in the DMSO- and DHA-treated groups were observed. (D) Treatment with DHA resulted in significant inhibition of tumor volume. * $P < 0.05$, ** $P < 0.01$ vs. DMSO-treated control (n=6). DHA, dihydroartemisinin; DMSO, dimethyl sulfoxide.

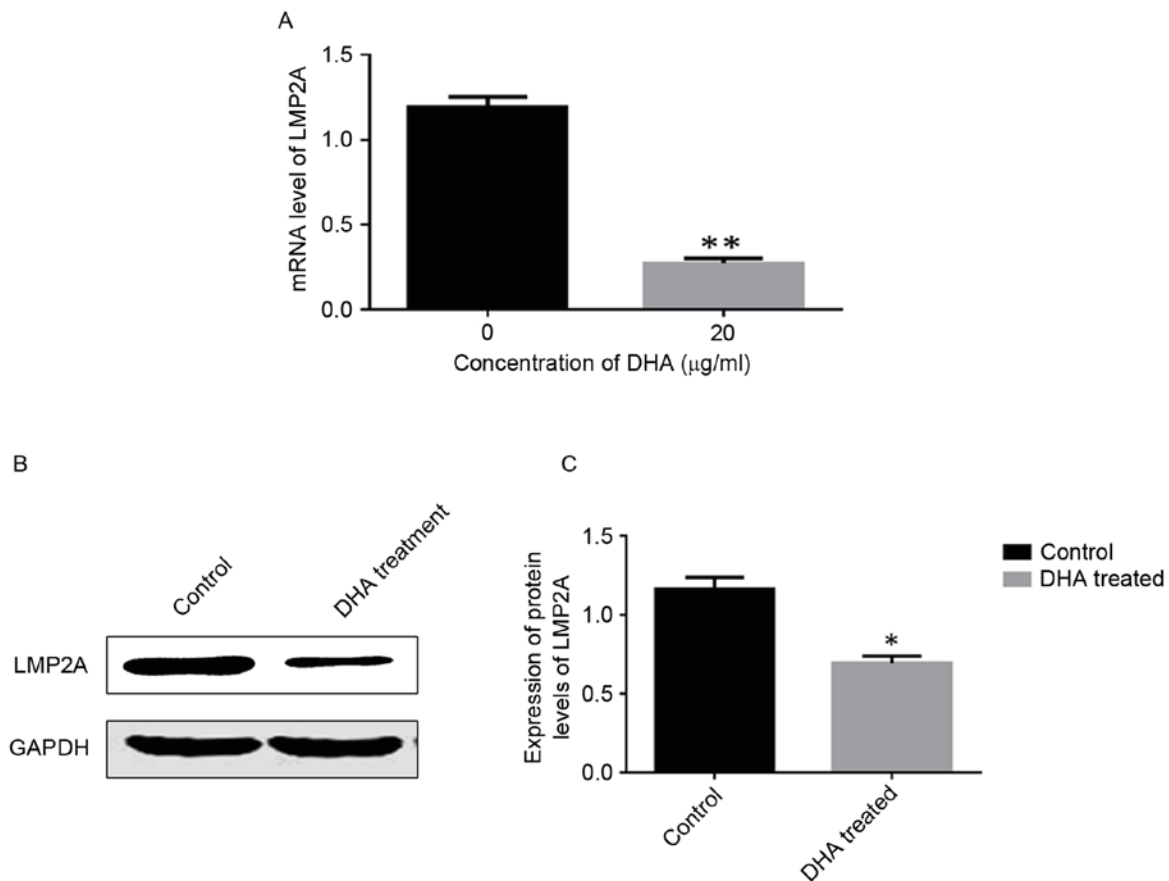


Figure 4. LMP2A mRNA and protein levels in GT38 cells. (A) The relative mRNA level of LMP2A compared with the control group was detected by reverse transcription-polymerase chain reaction following 0 and 20 $\mu\text{g/ml}$ DHA treatment. (B) LMP2A protein levels in the different groups was assessed by western blotting. (C) The relative protein level of LMP2A compared with GAPDH of each group. * $P < 0.05$, ** $P < 0.01$ vs. control ($n = 3$). LMP2A, latent membrane protein 2A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

DHA treatment downregulates LMP2A expression in GT-38 cells. The RT-PCR results revealed significantly lower LMP2A mRNA expression levels in DHA-treated cells compared with untreated cells ($P < 0.05$; Fig. 4A). Western blotting results demonstrated that the protein expression levels of LMP2A were significantly lower in DHA-treated cells compared with control cells. ($P < 0.05$; Fig. 4B and C).

Discussion

The morbidity and mortality of gastric cancer has decreased in developed countries due to improved prognosis for early stage cancer (17). However, it remains the second leading cause of cancer-associated mortality globally (17). EBVaGC comprised 10% of gastric carcinoma cases in 2014, which is estimated to exceed 75,000 cases/year across the world (18). Treatment of recurrent and metastatic disease remains a challenge, particularly in developing countries.

DHA, a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *A. annua*, is used as a first-line anti-malarial drug with low toxicity (19). In previous years, artemisinin and its derivatives have been identified as a promising drug to induce cancer cell apoptosis and inhibit viral infection (20). It was reported that DHA may inhibit pancreatic cancer cells *in vitro* through downregulating the expression of proliferating cell nuclear antigen and

cyclin D1, upregulating cyclin dependent kinase inhibitor 1 expression and inducing apoptosis by reducing the ratio of B-cell lymphoma-2 (Bcl-2)/Bcl-2 associated X protein and increasing the activation of caspase-9 in a dose-dependent manner (9). It was also reported that DHA inhibited pancreatic cancer cells in subcutaneous BxPC-3 xenograft tumors in mice (9). Furthermore, DHA may also inhibit cervical cancer cells by upregulation of kinase inhibitor protein, a suppressor of metastasis, and downregulation of Bcl-2 (10). In addition, Sun *et al* (21) reported that the proliferation rate and colony-forming abilities of gastric cancer cells were significantly suppressed by DHA, together with significant suppression of the expression of proliferation markers (proliferating cell nuclear antigen, cyclin E and cyclin D1) and the upregulation of p21 and p27. However, it remains unknown whether artemisinin and its derivatives work as growth inhibitors in EBVaGC cells.

The present study investigated the inhibitory effect of DHA on EBVaGC cells. The present data demonstrated that DHA significantly inhibited the viability of EBVaGC cells in a dose-dependent and time-dependent manner. This was in accordance with previous studies, which also demonstrated a dose-dependent and time-dependent inhibition of different types of cancer cell viability following DHA treatment (9-11). The present results also demonstrated that DHA significantly increased the apoptotic rate of GT-38 cells. The same cancer

cell inhibitory effect of DHA was also reported by Lee (22), and they reported that DHA was effective in inhibiting ovarian cancer cell proliferation at doses of micromolar levels and in a time-dependent manner. The present results also demonstrated that DHA treatment altered the cell cycle profile of GT38 cells, resulting in a significant increase in the percentage of G₀/G₁ phase cells. As more GT38 cells were arrested in the G₀/G₁ phase, the potency of cancer cells to divide and proliferate was decreased. Another study also reported that DHA may suppress the growth of rhabdomyosarcoma cells through arresting the cell cycle at the G₀/G₁ phase (23).

LMP2A is an EBV-encoded transmembrane protein, which functions in numerous signal transduction pathways and is involved in EBVaGC tumorigenesis (12,24). To understand the mechanism of DHA on the apoptosis of EBVaGC cells, the effect of DHA on *LMP2A* expression was studied. The present data revealed that the mRNA and protein levels of *LMP2A* were significantly downregulated in the DHA-treated group compared with the control group. *LMP2A* may be detected in the majority of EBVaGC and functions in maintaining latent pattern, promoting viral gene expression, inhibiting apoptosis and serving an important function in the pathogenesis of EBVaGC (13-15,25-27). It was observed that DHA downregulated them RNA and protein levels of *LMP2A*, and the decreasing *LMP2A* expression induced the observed cell viability inhibition and cell apoptosis of EBVaGC cells. DHA may therefore inhibit cell viability and induce cell apoptosis of EBVaGC cells via downregulating the expression of *LMP2A*. It was reported that *LMP2A* upregulates the expression of survivin, which confers resistance to serum deprivation-induced apoptosis in EBV-infected gastric carcinoma cells (15). However, additional studies are required to investigate the mechanism of how *LMP2A* mediates the suppressing effect of DHA on GT-38 cell viability.

In summary, the present study demonstrated that DHA may significantly suppress cell growth and induce apoptosis in EBVaGC cells by downregulating *LMP2A* expression. This provides an attractive therapeutic candidate for treating human EBVaGC.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XLW and QL conceived and designed the study. WG, LZ, HY, WKP, YW and QY performed the experiments. WG wrote the paper. WKP, YW, XLW and QL reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The animal use protocol was approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University Second Affiliated Hospital (Xi'an, China).

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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