Original Article
Human cytomegalovirus infection enhances invasiveness and migration of glioblastoma cells by epithelial-to-mesenchymal transition

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Abstract: Objective: This study aims to investigate the effect of human cytomegalovirus (HCMV) infection on epithelial-to-mesenchymal transition (EMT) in glioblastoma cells and the possible underlying molecular mechanism. Methods: We established primary cell cultures and measured the expression of the HCMV immediate early protein (IE1) to determine HCMV infection by immunohistochemical assays. Human glioma cells were divided into four groups: primary HCMV-positive, primary HCMV-negative, HCMV-positive U87, and HCMV-negative U87 cells. Cells were treated with transforming growth factor (TGF-β1, 5 ng/ml) to induce EMT. Morphologic changes of the cells were observed microscopically at 0, 24, 48, and 72 h post TGF-β1 treatment. Following EMT induction, E-cadherin and vimentin were detected using RT-PCR. Expression of MMP-2, E-cadherin, and vimentin was measured by western blotting. The invasiveness of glioma cells was also measured using the Transwell migration assay and a wound-healing assay. Results: Morphologic changes in primary glioblastoma cells and U87 cells were observed at different times after exposure to TGF-β1, and the extent of these changes was greater in HCMV-positive compared with HCMV-negative cells. Following exposure to TGF-β1, the transcription of E-cadherin was significantly lower in HCMV-positive primary cells and U87 cells compared with HCMV-negative cells (P<0.01), which was consistent with the results of western blotting. The expression levels of vimentin were also elevated in HCMV-positive cells at 48 and 72 h. HCMV-positive U87 cells were significantly more invasive and migratory than HCMV-positive primary cells. TGF-β1 and HCMV were observed to accelerate EMT and cell invasion by the Jun N-terminal kinase (JNK) pathway. Collectively, our findings indicate that HCMV and TGF-β1 promoted cell invasion and migration in glioma cells by the JNK pathway. Conclusion: HCMV infection can promote EMT and strengthen the invasiveness of glioma cells.

Keywords: Human cytomegalovirus, glioblastoma, epithelial-mesenchymal transition, transforming growth factor-β1

Introduction

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults and accounts for 40%-50% of central nervous system tumors [1]. A rapid disease progression, high invasiveness and diffuse tumor growth, and recurrence after surgical resection remain major challenges for patient survival.

Human cytomegalovirus (HCMV) has been detected in various types of tumors, including glioblastoma, medulloblastoma, neuroblastoma, as well as prostate, breast, colorectal, and ovarian cancers [2]. Recent clinical epidemiological data illustrate that more than 90% of malignant gliomas are associated with human cytomegalovirus (HCMV) infection [3-6]. After Cobbs et al. [4] detected varying levels of HCMV genes and proteins in glioblastoma tissues (levels II-IV) for the first time, Priel et al. also demonstrated that the HCMV genome and encoded proteins exist in more than 64% of glioblastoma tissues [5]. Cobbs et al. transferred the HCMV genome into glioblastoma cells and observed that the development and aggressiveness of tumor cells was enhanced [4]. Thus, HCMV infection may play a major role in the occurrence and development of glioblastoma. However, the exact role of the HCMV in tumor development or invasiveness remains uncertain.

The mechanism of the invasive growth of glioblastomas is controversial [5, 7]. Growing morphologic and molecular evidence shows that epithelial-mesenchymal transition (EMT) plays an important part in the malignant progression of many tumor types [8, 9]. EMT, mainly manifested as changes in cell phenotype, is a critical cellular procedure deemed important for embryonic development, wound-healing, tumorigenicity, and malignant progression. During the course of EMT, the interaction between cells and the extracellular matrix is reconstructed, which results in the separation of epithelial cells from each other and from the underlying basement membrane, and also activates new transcriptional processes toward promoting the expression of mesenchymal cells. In tumor-producing environments, EMT leads to an increased initial motility and metastatic potential of cancer cells, and is selected to display a stronger anti-elimination ability through various regimens [10, 11]. Epithelial cells are thought to gradually lose adhesion under specific induction factors following which the E-cadherin-based cytoskeleton is converted into a vimentin-based cytoskeleton. This phenotypic transformation and loss of adhesion, allows tumor cells to become more invasive.

EMT is a dynamic process that can be triggered by stimulation from the microenvironment, including those from the extracellular matrices and secreted soluble factors/cytokines such as the transforming growth factor β (TGF-β), epidermal growth factor (EGF), and hepatocyte growth factor. The transforming growth factor β (TGF-β) comprised of four subtypes TGF-β1, TGF-β2, TGF-β3, and TGF-β1β2, adjust cell growth and differentiation. TGF-β1 is most prevalent among the group, and its involvement in EMT and tumor metastasis has been confirmed [12-14]. TGF-β1 is produced by the stromal cells surrounding the glioma cells, and glioma cells can also secrete this cytokine. Thus, TGF-β1 can govern the multiplication and differentiation of tumor cells through paracrine or autocrine mechanisms. Zhang et al. [15] discovered that the TGF-β1 was expressed in 93.8% of advanced glioblastomas and 43.1% of low-grade gliomas, respectively, and its expression was positively related to the microvessel density. Studies in vitro [16] have also suggested that TGF-β1 stimulation could significantly enhance the invasiveness of U251

and SHG44 glioma cells. Other studies [17, 18] indicated that TGF-β1 was a major immunosuppressive factor secreted by human glioma cells, which can exert biologic effects in combination with the TGF-β1 receptor in vivo. However, the relationship between TGF-β1 levels and glioma malignancy levels is unclear. Kjellman et al. [19] used immunohistochemistry and assessed samples from 23 patients with gliomas and found a positive correlation between TGF-β1 levels and the severity of gliomas.

Tabata et al. [20] demonstrated that infection with HCMV can induce activation of TGF-β1 in endotheliocytes through an integrin-mediated mechanism. Many studies in vivo and in vitro have discovered that HCMV infection promotes the secretion and activation of TGF-β1 [14]. There is less relevant literature on the role of human cytomegalovirus infection in EMT of other cancer. A study by Wan showed that human cytomegalovirus infection enhanced EMT by downregulating E-cadherin and upregulating the N-cadherin, fibronectin, and vimentin in colorectal cancer-derived stem cell-like cells [21]. Epithelial-mesenchymal transition (EMT) and its reversal process (mesenchymal-epithelial transition, MET) run through the invasion and metastasis of tumors. EMT is the initial link of cancer metastasis. Cell adhesion is reduced, and its mobility and invasiveness are enhanced, which helps tumor cells to escape from the primary focus and enter the surrounding blood vessels or lymphatic system. Under the influence of the microenvironment, tumor cells undergo MET and restore the epithelial phenotype and the ability to adhere, which is conducive to the "homing" and proliferation of tumor cells to form metastases. Oberstein et al. discovered that HCMV infection could inhibit EMT and induce MET in two mesenchymal breast cancer lines [18].

However, it has not been explicitly reported whether HCMV infection can promote EMT in glioblastoma and increase its invasiveness. In this study, primarily cultured glioblastoma cells were coped with TGF-β1 to induce EMT, following which epigenetic and molecular methods were used to investigate whether infection with HCMV plays a synergistic role in glioblastoma progression. This study will help to elucidate the complexity of HCMV pathogenesis and better understand the relationship between HCMV infection and glioblastomas, as well as

Table 1. Primer sequences for RT-PCR

gene	Primer Sequence	Product length
E-cadherin	Forward: 5'-CCAGTTTTCTCGTCC ATGCC-3'	135 bp
	Reverse: 5'-CACTTTCAGCCAGCCTGTCT-3'	
vimentin	Forward: 5'-TCCAGCAGCTTCCTGTAGGT-3'	197 bp
	Reverse: 5'-CCCTCACCTGTGAAGTGGAT-3'	
β-actin	Forward: 5'-TCGTGGGCCGCTCTAGGCAC-3'	165 bp
	Reverse: 5'-TGGCCTTAGGGTTCAGGGGG-3'	

the molecular mechanisms underlying EMT. This information will ultimately aid in improving the treatment of glioblastoma and preventing its recurrence.

Materials and methods

Primary glioblastoma cell culture

Primary glioblastoma cells were harvested from two male patients (37 and 48 years old) pathologically diagnosed with grade IV of glioblastoma in the Department of Neurosurgery, Affiliated Hospital of Qingdao University. The pieces of tumor tissue (2-3 mm size) were digested with trypsin for ten minutes, and then washed with D-Hanks solution. DMEM culture medium supplemented with 15% fetal bovine serum (FBS) was added to the Petri dishes containing tissue blocks, and placed in an aseptic incubator at 37°C under 5% CO_2 for 6 h. Medium was changed every 24-36 hours, meanwhile the observation was made 2-3 times a day, and photos were recorded. Cells were digested and passed for 2 to 3 generations to the required quantity.

Cell culture and infection with virus

U87 cells (American Type Culture Collection) were cultured in minimal medium. All cells were incubated in 20% FBS (GIBCO, USA) enriched with 1% penicillin/streptavidin (GIBCO, USA) at 37°C in 5% CO_2 atmosphere. HCMV AD169 (France Pasteur Laboratory, Paris, France) was tittered by plaque titration and expressed as the number of plaque-forming units per milliliter. Glioma cell lines were infected at a multiplicity of infection of 10 [22].

Immunofluorescence assays

Glioblastoma cells in the logarithmic growth phase were inoculated into a 24 well plate (density, 0.8 × 10⁵ cells/ml) and covered with lysine-coated glass slides at 37°C in a 5% CO_2 incubator for 24 h. Subsequently, cells were washed, fixed, and then blocked with 10% goat serum. After overnight incubation with mouse anti-IgE monoclonal antibody, the wells were washed and incubated in fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG for 2 h at 37°C. Cells were stained with Hoechst dye at room temperature for 10 min, and images were obtained using a laser-scanning confocal microscope (FV10i, Olympus).

RNA extraction and quantitative RT-PCR analysis

Logarithmically growing glioblastoma cells from all three groups were inoculated into cell culture dishes (density, 0.8 × 10⁵ cells/ml) and cultured in DMEM/F12 supplemented with 2% FBS for 48 h at 37°C in a 5% CO_2 atmosphere and saturated humidity. RNA extraction respectively was carried out after 24, 48 and 72 hours using a mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA, USA). The cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) with appropriate primers using an ABI 7500-fast thermocycler (Applied Biosystems, Foster City, CA). Primers used for amplification are listed in **Table 1**. PCR amplification was performed as follows: 5 min at 95°C, 40 cycles of 10 s at 95°C, 40 s at 60°C, and 30 s at 40°C.

Western blot analysis

Total cellular protein was extracted from cells of three groups. After proteins were transferred to a PVDF membrane, the membranes were blocked with 5% nonfat dry milk at room temperature for 2 h and washed and incubated with the primary antibodies overnight at 4°C in a refrigerator. (Anti-E-cadherin, Immunoway; Anti-vimentin, Immunoway; Anti-MMP-2, Abcam; Anti-p-actin, Abcam; antibodies diluted to 1:1000). Membranes were washed and incubated in HRP-labeled secondary antibodies (Bioss) for 2 h at room temperature. After washing, they were incubated in a developing solution for 10 min and then imaged using an imaging system developed by Vilber Lourmat.

Transswell assay

The transwell migration assay kit was obtained from Millipore. The membrane in the bottom chamber was coated with 50 mg/l Matrigel (1:8 ratio) and air-dried under sterile conditions. For each cell group, a 200 μl suspension containing 1×10^4 cells was inoculated into the upper chamber. After incubation for 24, 48, and 72 h at 37°C in 5% CO_2 atmosphere, they were rinsed thrice with PBS, fixed with 2% paraformaldehyde for 10-15 min and stained with 0.1% crystal violet for 10 min. Cells were counted from six randomly recorded images.

Immunohistochemistry (IHC)

The main steps, used in a standard protocol, included immobilization, paraffinization, dewaxing, dehydration, antigen extraction, blocking, primary antibody incubation (HCMV IE1 antibody), washing, secondary antibody incubation, diaminophenyldiamine antigen-antibody reaction, and observation.

Wound healing assay

First, a horizontal line (0.5-1 cm) was evenly drawn behind the 6-hole plate, then cells were seeded in a 6-well plate (total number of cells ~ 5×10^5 were spread overnight on the plate). After scraping, cells were washed thrice with PBS and the delineated cells were added to a serum-free medium and cultured at 37°C in 5% CO_2 incubator. Samples were extracted after 24 h and pictures of microscopic examination were recorded.

Statistical analysis

Results were expressed as the mean +/- standard deviation (±s), and differences between the groups were analyzed using the Student's t-test and one-way analysis of variance. $P<0.05$ was considered significant. All analyzes were performed using version 21.0 of SPSS Statistics Software (SPSS Inc., Chicago, IL, USA).

Results

Primary cell culture

Two clinical glioma samples were analyzed and each sample was divided into two parts. One part was used for analyzing HCMV infection by immunohistochemistry whereas the other was used for primary cell culture. Immunohistochemical analyzes indicated one of the samples to be positive for HCMV and the other negative for HCMV (**Figure 1A**). Two primary glioma cell lines were established successfully from the two independent glioma samples (**Figure 1B**). Immunofluorescence and western blot assay confirmed one of the cell lines to be HCMV-positive and the other to be HCMV-negative (**Figure 1C-E**).

Cell morphology

As shown in **Figure 1F**, primary glioblastoma cells and U87 cells were assessed for morphologic changes at 24, 48, and 72 h after exposure to TGF-β1. Morphologic changes in HCMV-positive cells were greater when compared to HCMV-negative cells. Prior to TGF-β1 exposure, the glioma cells exhibited a stone-like, single layer adherent growth with close intercellular connections. However, following exposure to TGF-β1, the cells displayed a spindle-shaped morphology with a disorderly arrangement, looser connections, and larger gaps between cells.

Synergistic effect of HCMV with TGF-β1 on EMT

E-cadherin and vimentin are critical to EMT progression. To investigate the effects of HCMV and TGF-β1 on EMT in glioma cells, we examined the expression levels of E-cadherin and vimentin in the four different cell groups (HCMV-positive primary cells, HCMV-positive primary cells, HCMV-positive U87 cells, and HCMV-negative U87 cells). The transcription levels in these groups were examined by RT-PCR and results are shown in **Figure 3**. Following exposure to TGF-β1, the transcription of E-cadherin was significantly lower in HCMV-positive primary cells and U87 cells compared with HCMV-negative primary cells and U87 cells (**P<0.01) (**Figure 2A**). These results were consistent with those observed using western blotting (**Figure 2B**). Similarly, the mesenchymal cell marker vimentin was expressed at 24 h in both the HCMV-positive cells groups compared with the HCMV-negative cell groups. The levels of vimentin expression were also elevated in HCMV-positive cells at 48 and 72 h (**Figure 2B-D**). Moreover, we also detected an expression of
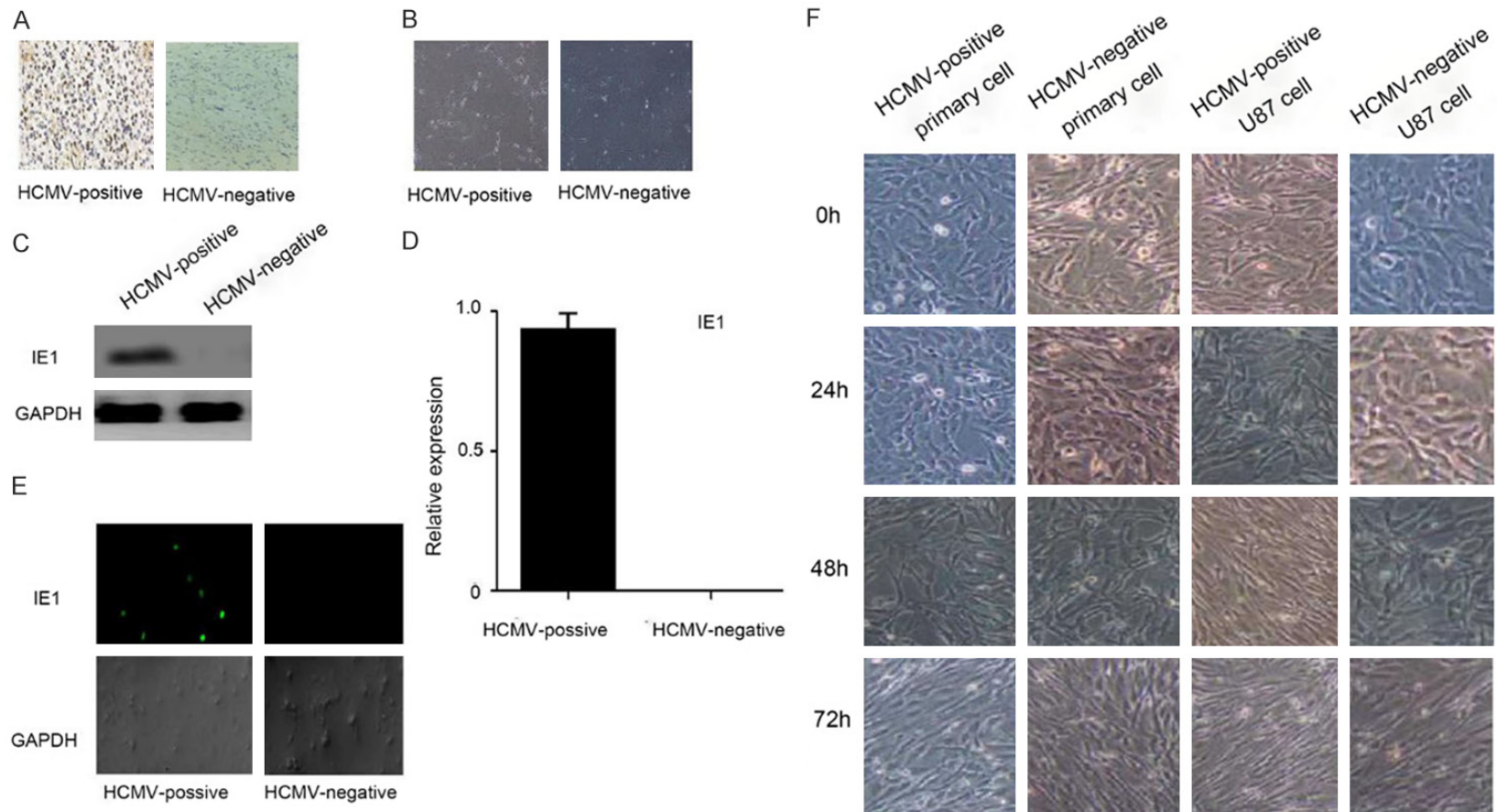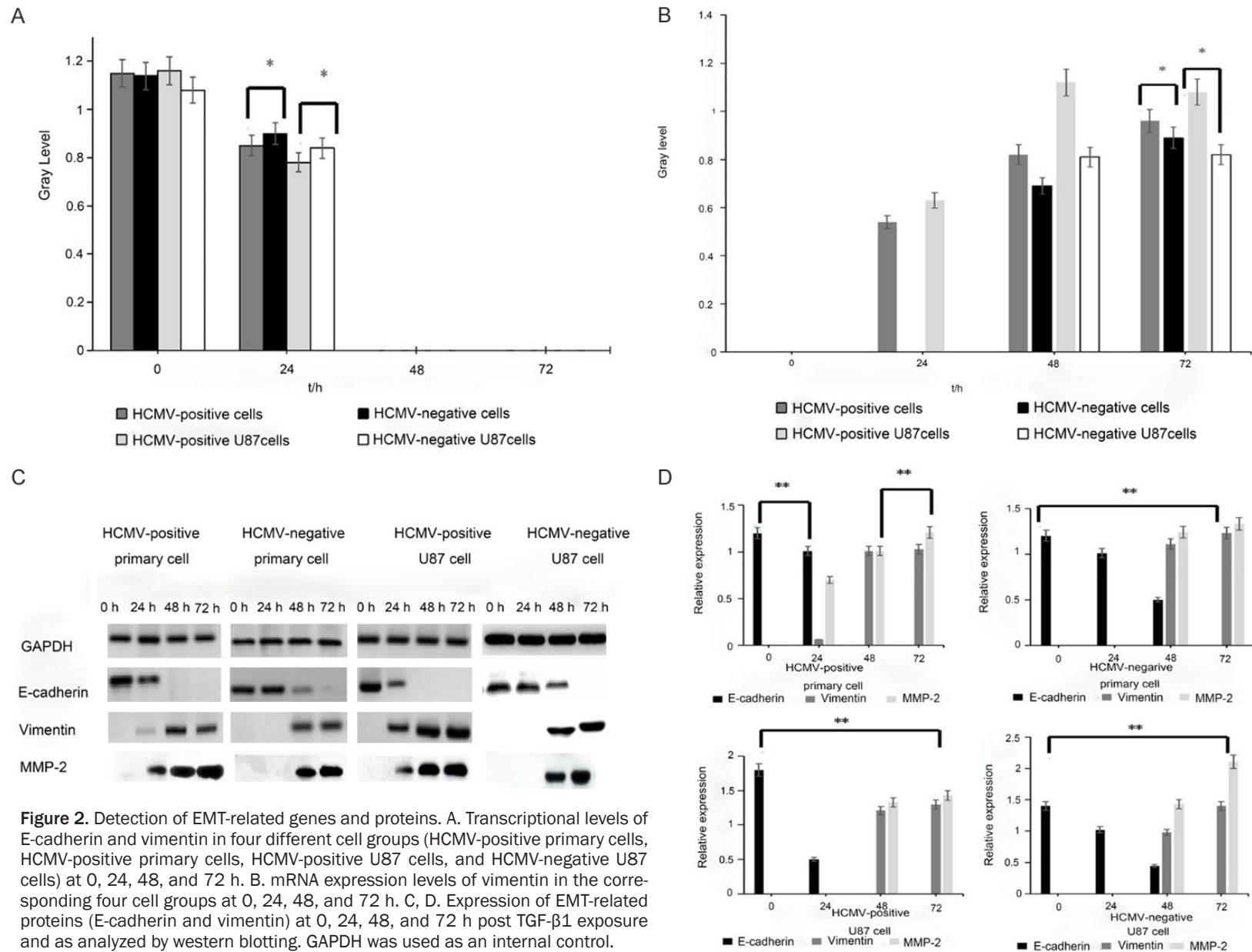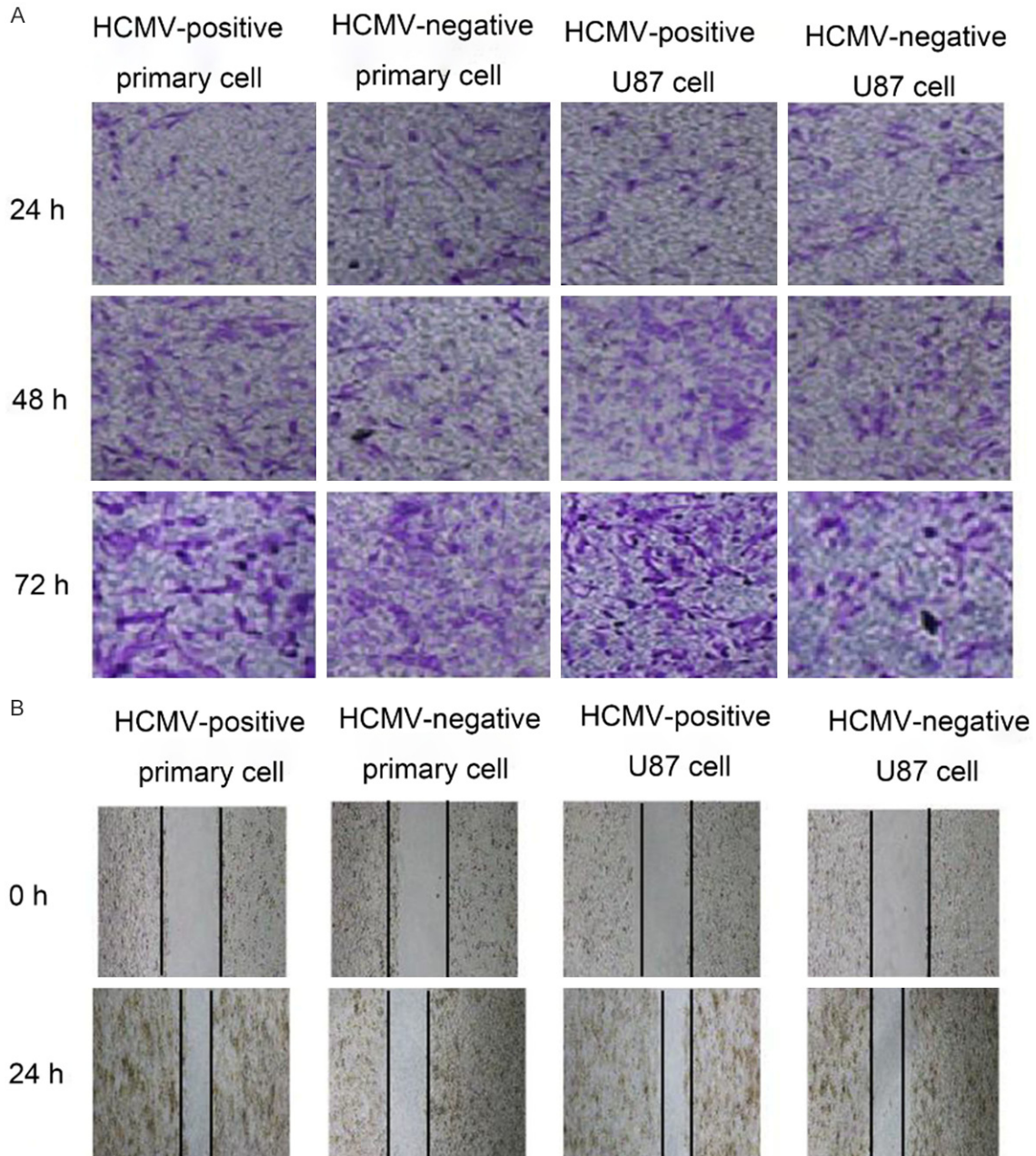
Figure 1. HCMV-infected in primary glioblastoma cells. A. Immunohistochemical analysis indicated one sample as HCMV-positive (left panel) and other as HCMV-negative (right panel). B. Primary cell lines were established using the HCMV-positive and-negative cell lines and were observed using CLSM. HCMV-positive and-negative samples were confirmed by western blotting. C, D. Using antibodies against IE1, GAPDH was used as internal reference standard for western blotting. E. Immunofluorescence staining also confirmed the presence or absence of HCMV in the two different cell lines. F. Morphologic changes in primary glioblastoma cells and U87 cells after 0, 24, 48, and 72 h of exposure to TGF-β1 (scale bar = 100 μm).

Figure 2. Detection of EMT-related genes and proteins. A. Transcriptional levels of E-cadherin and vimentin in four different cell groups (HCMV-positive primary cells, HCMV-positive primary cells, HCMV-positive U87 cells, and HCMV-negative U87 cells) at 0, 24, 48, and 72 h. B. mRNA expression levels of vimentin in the corresponding four cell groups at 0, 24, 48, and 72 h. C, D. Expression of EMT-related proteins (E-cadherin and vimentin) at 0, 24, 48, and 72 h post TGF-β1 exposure and as analyzed by western blotting. GAPDH was used as an internal control.

Figure 3. Detection of the invasive and migratory ability of glioblastoma cells. A. Transwell assay was performed to detect cell invasion ability. B. Wound-healing assay was performed to detect cell migration ability.

MMP-2, suggesting that MMP-2 and vimentin expression are in agreement (**Figure 2B**).

Migration assay and invasion assay in glioblastoma cells

Transwell experiments were performed to examine the effect of TGF-β1 and HCMV infection on the invasiveness of primary glioblastoma cells in vitro. The numbers of invading cells in the four analyzed cell groups were examined at 24, 48, and 72 h (**Figure 3**). The results illustrated that HCMV-positive U87 cells were significantly more invasive than HCMV-positive primary cells (*$P<0.05$). Further, the HCMV-positive cells were more invasive than their HCMV-negative counterparts (*$P<0.05$). The results corroborated those of the wound-healing assay.
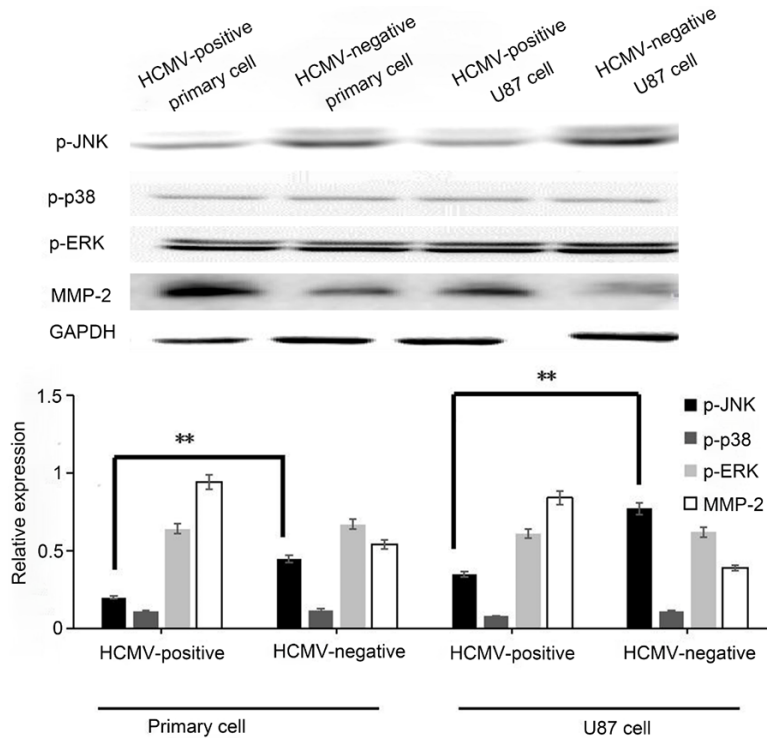
Figure 4. Western blot analysis of proteins obtained from four different cell groups that were either HCMV-positive or -negative. GAPDH was used as an internal control. TGF-β1 and HCMV promote cell invasion through the JNK pathway.

TGF-β1 and HCMV promote cell invasion through JNK pathway

In order to detect the underlying pathway by which TGF-β1 and HCMV play a role in cell invasion, we examined the expression of Jun N-terminal kinase (JNK), p38, and ERK proteins. We observed that the expression of JNK was changed upon exposure to TFG-β1. The expression of JNK was significantly downregulated simultaneously as the invasive ability of cells was observed to become stronger. Thus, upon infection with HCMV, JNK may probably play a synergistic role with TGF-p1 in promoting cell invasion. Other proteins showed no significant changes as observed in **Figure 4**. Therefore, we speculated that TGF-β1 and HCMV promote cell invasion by the JNK pathway.

Discussion

This study aimed at better understanding the presence of HCMV in glioblastomas (GBMs) by investigating the effect of HCMV infection on EMT in primary glioblastoma cells lines. To this end, we detected a downregulated expression of E-cadherin and an upregulated expression of vimentin and MMP-2 in the HCMV-positive glioblastoma cells lines. Based on this result, we concluded that HCMV infection may enhance invasion and migration of tumor cells through EMT.

EMT is a very complex molecular process. The epithelial tissue can "mask" the characteristics of differentiated cells, such as cell-cell adhesion, cell polarity, and cell lack of mobility by the process of EMT. This can lead to acquisition of characteristics of interstitial cells, such as motility, invasiveness, and resistance to apoptosis [23]. Upon occurrence of EMT, cells gradually lose the epithelial cell phenotype and obtain one characteristic of mesenchymal cells. Hereby, the expression of E-cadherin and cytoskeletal keratin is decreased, while the expression of vimentin, fibronectin, and N-cadherin is increased. Simultaneously, phenotypic changes associated with EMT are also regulated by signaling pathways such as the EGF receptor, Wnt and mitogen-activated protein kinase, which together promote cell movement and migration, and ultimately increase their invasiveness leading to distant metastasis of tumor cells [24].

In the current study, we selected TGF-β1 to induce EMT in HCMV-positive and -negative glioblastoma cells. Morphologic changes occurred in all cell groups of cells after TGF-β1 induced activation. However, morphologic changes in HCMV-positive cells were significantly greater than those in HCMV-negative cells. These phenotypic changes led tumor cells to gradually lose their adherence to epithelial tissue, resulting in increased cell invasion, escape, and transfer. Simultaneous morphologic changes that occurred in the HCMV-positive and -negative glioblastoma cells, may reflect the low HCMV titers in the former (HCMV-positive) group.

Further investigation indicated that TGF-β1 was capable of downregulating the expression of E-cadherin and upregulating that of vimentin and MMP-2 in HCMV-positive glioblastoma cells. E-cadherin is an epithelial cell marker, and its downregulation or loss signifies an important change in EMT. The extracellular region of E-cadherin can span the cell membrane, whereas its intracellular domain can form a variety of complexes with E-catenin and actin in order to achieve intercellular adhesion by forming strong physical connections between cells [25, 26]. Conversely, the upregulation of vimentin and MMP-2 indicate cell movement and matrix degradation, respectively [27, 28]. Increased vimentin expression can lead to cytoskeletal changes, which cause epithelial cells to lose polarity, thus transform them into fibroblasts with interstitial properties such as invasive ability and capacity to metastasis [19]. Results of the current study illustrated that TGF-β1 can collaborate with HCMV to induce EMT in primary glioblastoma cells and potentially strengthen their invasiveness. These results were confirmed by the Transwell assay, where the number of invasive cells was observed to be higher in case of HCMV-positive cells compared with HCMV-negative cells at each analyzed time point. Similar results were reported by Shimamura [29] who observed that renal tubular epithelial cells infected by HCMV can go through EMT after TGF-β1-induced activation, and cells infected with HCMV can promote the secretion of TGF-β1 through MMP-2. MMP-2 is a type IV collagenase, an important mediator of tumor-interstitial interaction. Its expression gradually increases during epithelial-mesenchymal transition of cells [12].

TGF-β signaling can induce EMT transformation by a number of signaling mechanisms, such as direct phosphorylation of SMAD transcription factor ligands to activate receptors, and through certain cytoplasmic factors that regulate cell polarity and tight junction formation between cellular proteins that trigger the EMT conversion process. TGF-β also affects the activity of several other EMT triggering signaling pathways, such as Notch, Wnt, and integrin signaling pathways.

The SMAD independent TGF-β pathways include mitogen-activated protein kinase (MAPK) pathways, Rho-like GTPase signaling pathways, phosphatidylinositol-3-kinase/AKT pathways, p38, and JNK pathways. Among the non-SMAD pathways, the JNK and p38 pathways have been reported to produce transcriptional modifications at cellular level necessary for EMT after induction with TGF-p [30, 31]. In the current study, we observed that HCMV and TGF-β1 promoted cell invasion and migration in glioma cells by the JNK pathway. JNK is a member of the MAPK family that regulates a range of biological processes implicated in tumorigenesis and neurodegenerative disorders. For example, genetic studies have demonstrated that the removal of specific JNK genes can reduce neuronal death associated with cerebral ischemia. As such, targeting JNK signaling constitutes an obvious opportunity for therapeutic intervention [32].

EMT plays an important role in the occurrence and development of tumors, which may be applied as a major indicator for detecting cancer. Since the mechanisms underlying EMT are extremely complex, there are currently no reliable methods for predicting the key transcription factors engaged in different tumors. Thus, it is significant to investigate the molecular mechanisms of EMT in the occurrence and development of cancer. This will also aid in clinical diagnosis, prognosis, and targeted treatment of cancer.

Compliance with ethics guidelines

This research was approved by the ethics committee of the Affiliated Hospital of Qingdao University and the Human Investigation Committee of Qingdao University. (Approval number: 2017049) There is no declarable conflict of interest among the authors.

Disclosure of conflict of interest

None.

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