

Human cytomegalovirus glycoprotein B inhibits migration of breast cancer MDA-MB-231 cells and impairs TGF- β /Smad2/3 expression

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Abstract. Breast cancer is a leading cause of cancer-associated mortality in females worldwide and evidence suggests that human cytomegalovirus (HCMV) infection may be implicated in the progress of breast cancer. HCMV glycoprotein B (gB) is the most abundant envelope protein and serves an important role in host cell entry. The present study aimed to clarify the role of HCMV gB in breast cancer cells. A HCMV gB construct (UL55) was generated and stable vUL55 gene lentivirus-transfected MDA-MB-231 cells were established. Subsequently, the effect of HCMV gB on the apoptosis and proliferation of MDA-MB-231 cells was measured by flow cytometry and Cell Counting Kit-8 assay. Furthermore, whether HCMV gB may modulate MDA-MB-231 cell migration was examined using Transwell and cell scratch assays. In addition, alterations in HCMV gB-modulated protein levels of transforming growth factor- β (TGF- β) and Mothers against decapentaplegic homologs 2/3 (Smad2/3) were detected using western blot analysis. The results indicated that UL55 cDNA was stably transfected into MDA-MB-231 cells, and that HCMV gB protein was stably expressed. No significant differences in cell apoptosis and proliferation between transfected (231-GB-OE) and negative control (231-NC) cells were observed, while the rate of cell migration was significantly decreased in the 231-GB-OE cells compared with the 231-NC cells. Additionally, the expression level of TGF- β

and phosphorylation level of Smad2/3 were also decreased in 231-GB-OE cells compared with the 231-NC cells. Although certain previous studies indicated that HCMV infection was associated with breast carcinogenesis, the results of the present study indicate that the envelope protein HCMV gB exhibits no effect on cell apoptosis and proliferation, but inhibits breast cancer cell migration. This may be due to downregulated TGF- β /Smad signaling. Taken together, these studies may assist in developing anti-TGF- β agents that contribute to tumor suppression.

Introduction

Human cytomegalovirus (HCMV), a member of the beta-herpesvirinae (HHV) sub-family, is a widespread viral agent that infects 50-90% of people worldwide (1). Following primary infection, in which the production of infectious viruses is undetectable and viral gene expression is highly restricted (2), HCMV establishes a lifelong latency in cells of the myeloid lineage (3). It may also transmit from mother to fetus and results in a disease burden that is substantial and severe, including sensorineural hearing loss (4-6). In healthy individuals, infection of HCMV is usually asymptomatic, but it may lead to life-threatening diseases, with multi-organ involvement and frequent fatalities in patients with deficient immune systems (7,8). HCMV glycoprotein (gB) is the major component of the virus envelope and the most highly conserved glycoprotein of the human HHV family (9). It serves an essential role in the HCMV entry process by binding to membranous Integrin β -1 via its HCMV gB disintegrin-like domain, which mediates virus entry to the host cell and cell-cell virus transmission (10).

Breast cancer is considered a leading cause of cancer-associated mortality and the most common malignancy among US women in 2013 (11). Although it is clear that age, estrogen level, family history and factors associated with lifestyle and diet are prominent risk factors for the onset of breast cancer (12), studies suggest that viruses maybe an additional high-risk factor closely associated with human breast cancer (13). For example, infection with human papillomavirus,

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Epstein-Barr virus, HCMV and HHV-8 has been suggested as risk factors or associated with the development of breast cancer (14). Among these viruses, HCMV was identified in patients with newly-diagnosed (15) and metastatic breast cancer (16). In addition, HCMV proteins and DNA have been identified in breast ductal carcinoma *in situ* and infiltrating ductal carcinoma tissues, suggesting that HCMV infection may be associated with breast carcinogenesis (17). HCMV gB, the most abundant and highly antigenic viral envelope protein (9), serves an important role in host cell entry, cell-cell virus transmission and fusion of infected cells (18). Previous data have demonstrated that HCMV gB may promote the growth, migration and infiltration of glioma by binding and activating platelet-derived growth factor receptor α (PDGFR α) and its downstream signaling pathways; therefore, targeting HCMV gB may have therapeutic benefits for patients with HCMV-positive tumors (19). However, it is not clear whether HCMV gB serves roles in the development of breast cancer.

Transforming growth factor- β (TGF- β) is secreted abundantly by tumors cells, and serves roles within complex bidirectional interactions in epithelial carcinogenesis (20,21). During breast cancer progression, TGF- β inhibits cell proliferation in well-differentiated, early stage breast tumors through the induction of cell cycle arrest and apoptosis (22-24), while in poorly differentiated advanced-stage breast tumors, these functions were replaced by tumor-promoting and pro-metastatic responses (25). The binding of TGF- β to its receptors leads to the recruitment and phosphorylation of Mothers against decapentaplegic homologs 2/3 (Smad2/3), and the majority of the pro-metastatic functions of TGF- β are attributed to the TGF- β /Smad signaling pathway (26,27). As Smad proteins are not ideal drug targets due to their roles as transcription factors, anti-TGF- β therapies have demonstrated potential in preventing the development of breast cancer and other types of cancer, including melanoma (28-30).

In the present study, to clarify the role of HCMV glycoprotein B (gB) in breast cancer cells, stable HCMV gB-transfected MDA-MB-231 cells were established, and it was identified that although HCMV Gb exhibited no effect on MDA-MB-231 cell proliferation and apoptosis, it led to the suppression of cell migration. In addition, it was demonstrated that HCMV gB inhibited TGF- β protein expression and Smad2/3 activation in MDA-MB-231 cells. To the best of our knowledge, these data are the first to demonstrate that HCMV gB suppresses breast cancer cell migration and inhibits TGF- β /Smad signaling; this may assist in developing novel anticancer agents that contribute to tumor suppression.

Materials and methods

Materials. The human MDA-MB-231 cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and maintained in a humidified atmosphere containing 5% CO₂-humidified atmosphere at 37°C. Western blot assays were performed using the following primary antibodies: Rabbit anti-human caspase-3

(cat. no. 14220; dilution, 1:1,000); rabbit anti-human caspase-9 (cat. no. 9508; dilution, 1:1,000); rabbit anti-human B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. 2772; dilution, 1:1,000); rabbit anti-human TGF- β (cat. no. 3711; dilution, 1:1,000); rabbit anti-human Smad2/3 (Smad2: cat. no. ab1305; Smad3: ab28379; dilution, 1:1,000); rabbit anti-human Bcl-2 (cat. no. 3498; dilution, 1:1,000); and mouse anti-actin (all from EMD Millipore, Billerica, MA, USA; cat. no. MAB1501; dilution 1:10,000). The secondary antibody HRP-conjugated antibodies [HRP-anti-rabbit antibody (cat. no. 7074; dilution, 1:10,000) and HRP-anti-mouse antibody (cat. no. 7076; dilution, 1:10,000)] were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The BCA protein assay kit was sourced from Beyotime Institute of Biotechnology (Haimen, China), the Cell Counting Kit-8 (CCK-8) from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan) and the apoptosis detection kit from BD Pharmingen (BD Biosciences, Franklin Lakes, NJ, USA). The 0.25% Trypsin-EDTA (1X) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc.), and the Transwell inserts were purchased from Corning Incorporated (Corning, NY, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). An equal amount of total RNA was used for first-strand cDNA synthesis using the oligo-dT primer and M-myeloblastosis virus reverse transcriptase XL (Promega Corporation, Madison WI, USA). The synthesized first-strand of cDNA (2 μ l) was used for each PCR. The target fragment and primer (UL55 forward, CCGCTC GAGGCCACCATGGAAT and reverse, CGGGATCCGACG TTCTCTTCTTC) were designed and synthesized in Sangon Biotech Co., Ltd. (Shanghai, China), and the qPCR analysis was performed using the GoTaq qPCR Master Mix (Promega Corporation), and GAPDH was used as an internal reference control. PCR was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 55°C for 1 min. Each reaction was performed in triplicate. Then, the product was ligated into the lentiviral vector PHY-022. The fast growing bacteria DH5 α were treated with 0.1 M CaCl₂ solution at 0°C for 20 min to obtain the receptive bacteria. The ligation product was transferred into the prepared receptive bacteria DH5 α , and the positive clones were identified by qPCR. The correct clones were those containing the lentiviral vector required (Fig. 1A).

Lentiviral vector construction and stable transfection of MDA-MB-231 cells. Lentiviral vector for gB (UL55) expression sequence were constructed by Hanyin Co (<http://www.hanyinbt.com>). (Shanghai, China). Prior to construction, a flag (GACTACAAGGACGATGACGACAAG TGA) used as a label of expression was added to the end of the UL55 sequence for tracking effect. The recombinant lentivirus and the negative control (NC) lentivirus (Hanyin Co.) were prepared and titered to 10⁹ TU/ml (transfection unit). To obtain the stable cell line, 2x10⁴ MDA-MB-231 cells were seeded in 6-well plates and incubated overnight in DMEM supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and maintained in a humidified atmosphere with 5% carbon dioxide-humidified atmosphere at 37°C. The

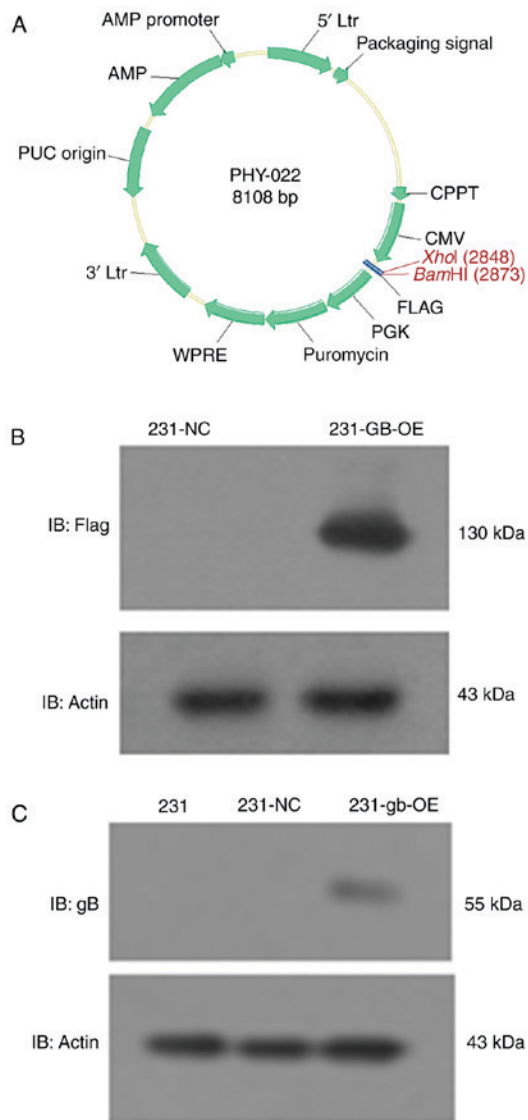


Figure 1. HCMV gB is successfully transfected into MDA-MB-231 cells. (A) Lentiviral vector structure. (B) Flag was expressed in 231-GE-OE cells. (C) Normal MDA-MB-231 cells (231) and the empty vector transfected cells (231-NC) did not express HCMV gB, while HCMV gB-transfected MDA-MB-231 cells (231-GB-OE) demonstrated gB expression; IB, immunoblotting; NC, negative control; AMP, Adenosine 5'-monophosphate; Ltr, long terminal repeat; WPRE, Woodchuck Hepatitis Virus posttranscriptional regulatory element; CPPT, central polypurine tract; CMV, cytomegalovirus; HCMV, human CMV; PGK, phosphoglycerate kinase promoter plasmid; PUC, origin of replication; gB, glycoprotein B.

cells exhibited 30-50% confluence at the time of transduction. The recombinant and negative control lentivirus particles were dissolved on ice, the DMEM was removed from the cells, and then the recombinant and negative control lentivirus/polybrene mixture medium [20 μ l of lentiviral particles and 8 μ g/ml polybrene (Sigma Aldrich; Merck KGaA, Darmstadt, Germany)] was added and the two group cells [cells transfected with the recombinant lentivirus (231-GB-OE) and the negative control (NC) lentivirus (231-NC)] were incubated for 72 h in a humidified atmosphere with 5% CO₂-humidified atmosphere at 37°C. The lentivirus/polybrene mixture medium was exchanged with fresh DMEM, and the 231-GB-OE and 231-NC cells were incubated for at least 5 days at 37°C. Subsequently, 231-GB-OE and 231-NC cells were collected and lysed after 5 days, and

HCMV gB expression was detected by western blot analysis of the flag added in the end of UL55 sequence. For the selection of viable cells, 2 μ g/ml puromycin (Sigma Aldrich; Merck KGaA) was added to the culture medium on day 3.

CCK-8. To test cytotoxicity, MDA-MB-231 cells with recombinant lentivirus (231-GB-OE) and the negative control (231-NC) cells (3,000 cells/well) suspension was prepared. A total of 100 μ l cell suspension was dispensed into a 96-well plate. The plate was pre-incubated in the humidified incubator at 37°C and 5% CO₂. After 24, 48, 72, 96 and 120 h, 10 μ l CCK-8 solution was added to each well of the plate consecutively, and the plate was incubated at 37°C and 5% CO₂ for an additional 2 h. The absorbance was measured at 450 nm using a microplate reader.

Transwell migration assay. The 231-GB-OE and 231-NC cells were cultured in serum-free DMEM in the humidified incubator at 37°C and 5% CO₂ for 12 h. After 12 h serum starvation, the medium was removed from the serum-free culture and cells were rinsed with PBS 3 times, harvested and the cell concentration was diluted to 1x10⁵/ml with DMEM. A total of 100 μ l cells were seeded in the upper chamber, and 600 μ l medium with 10% FBS was added to the lower chamber. The cells were incubated at 37°C and 5% CO₂ for 48 h. The medium was removed from the upper section, and the cells that had migrated through the membrane were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 30 min at room temperature, then rinsed 3 times with PBS to remove the excess crystal violet. A total of 6 fields of view were randomly selected under the light microscope (x100 magnification), then the number of cells was counted and the average calculated.

Cell scratch assay. The HCMV gB-transfected cells (231-GB-OE) and 231-negative control (NC) cells were seeded onto 6-well tissue culture plates in a humidified atmosphere containing 5% CO₂ at 37°C at a density of 1x10⁵/ml that after 24 h of growth they reached 70-80% confluence as a monolayer. The monolayer was gently and slowly scratched with a new 1 ml pipette tip across the center of each well. While scratching across the surface of the well, the long axial of the tip was perpendicular to the bottom of the well; the resulting distance of the gap was therefore equal to the outer diameter of the end of the tip. The well was gently washed 3 times with PBS to remove the detached cells. DMEM containing 2% FBS was added to the wells, and the cells were grown for an additional 24 h, following which images were captured by a camera under the light microscope (x10 magnification).

Cell apoptosis analysis. The 231-GB-OE and 231-NC cells were cultured in DMEM supplemented with 10% FBS. Cells were seeded (2x10⁴) onto 6-well tissue culture plates and incubated for 24 h at 37°C and 5% CO₂. The culture medium was removed, washed once with pre-warmed PBS and digested with 0.25% Trypsin-EDTA. The cells were collected into a 15-ml tube and centrifuged at 1,000 x g for 15 min at room temperature. The cells were then washed once with 500 μ l binding buffer (BD Biosciences), the 200 μ l binding buffer containing 5 μ l Annexin V-fluorescein isothiocyanate (FITC; BD

Biosciences) was added to the tube and the cells were stained for 10 min in the dark at room temperature. Subsequently, 5 μ l propidium iodide (PI) was added for an additional 5 min at room temperature. A total of 300 μ l binding buffer was added to the tube, and cell apoptosis was analyzed using Cell Quest software on a BD FACSAria flow cytometer (BD Biosciences).

Western blot analysis. The cells were lysed with 150 μ l/well RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate) containing protease inhibitors (Roche, Complete Mini) (www.roche.com.cn), and the protein concentration for each cell lysate was determined through BCA method (BCA kit; Beyotime Institute of Biotechnology). A total of 20–30 μ g protein were loaded into the wells of the 10% SDS-PAGE gel, following which the proteins were transferred from the gel to the polyvinylidene fluoride membrane and blocked for 1–2 h at room temperature with 5% skim milk powder in TBS and 0.05% Tween-20 (TBST). The membrane was incubated with primary antibodies (rabbit anti-human caspase-3, rabbit anti-human caspase-9, rabbit anti-human Bax, rabbit anti-human TGF- β), rabbit anti-human Smad2/3, rabbit anti-human Bcl-2 and mouse anti-actin) overnight at room temperature, washed three times with TBST and incubated with conjugated secondary antibody (HRP-anti-rabbit antibody and HRP-anti-mouse antibody) at room temperature for 2 h. The membrane was then placed in a cartridge and electrochemiluminescence reaction solution (cat. no. PI32209; Pierce; Thermo Fisher Scientific, Inc.) was added for 1–3 min, followed by exposure with X-ray in the darkroom at room temperature for 1 min. Resulting bands were analyzed with Image J software 1.4.3.67 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data were expressed as the mean \pm standard deviation and statistical differences or comparisons between two groups were assessed by paired Student's t-test using SPSS for Windows v.17.0 (SPSS, Inc., Chicago, IL, USA). Three repeat experiments were performed for each protocol. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HCMV gB is expressed in MDA-MB-231 cells. The constructed gB overexpression vectors were sequenced prior to transfection into MDA-MB-231 cells, and no genetic variation was confirmed within the wild gB gene sequence (data not shown). Then, the whole gene of HCMV gB was transfected into MDA-MB-231 cells by the lentiviral vector PHY-022 (Fig. 1A), and the levels of HCMV gB protein were determined by western blot analysis. Fig. 1B indicates that the UL55-flag which tagged HCMV gB was stably expressed in the 231-GB-OE cells. In addition, 231-GE-OE demonstrated gB expression while normal MDA-MB-231 cells (231) and negative control cells transfected with empty vectors (231-NC) did not (Fig. 1C).

Effect of HCMV gB on cell apoptosis. The flow cytometry analysis was used to confirm whether HCMV gB expression induced apoptosis in MDA-MB-231 cells. The 231-NC and 231-GB-OE cells were incubated in 6-well plates for 24 h,

and as demonstrated in Fig. 2A, PI Annexin V-FITC double staining revealed that there were no significant differences between 231-NC and 231-GB-OE cells in the percentage of apoptotic cells (Fig. 2B). These results indicated that HCMV gB did not induce apoptosis in the MDA-MB-231 cells.

Effect of HCMV gB on cell proliferation. To evaluate whether HCMV gB expression affects MDA-MB-231 cell proliferation, cell viability was determined by a CCK-8 cell proliferation assay. The absorbance at 450 nm, which has been directly associated with cell proliferation (31), indicates that cell proliferation in the 231-GB-OE cells exhibited no significant difference in comparison with the 231-NC cells (Fig. 3). This result suggests that HCMV gB has no effect on the growth of MDA-MB-231 cells.

HCMV gB inhibits cell migration. As HCMV gB expression has no effect on cell apoptosis and proliferation, Transwell and cell scratch assays were used to determine whether HCMV gB affected tumor migration. The results indicate that the wound-healing rate in 231-GB-OE cells was significantly decreased compared with that in the 231-NC cells at 24 and 48 h (Fig. 4A and B). To additionally validate this observation, cell migration was measured by a Transwell assay. The migration rates of 231-GB-OE cells to the lower part of the insert were significantly decreased following 48 h incubation compared with the 231-NC cells (Fig. 4C and D). Collectively, these results suggest that HCMV gB may inhibit the migration capacity of MDA-MB-231 cells *in vitro*.

Effect of HCMV gB on caspase-3, caspase-9, Bax, Bcl-2 and TGF- β expression. To confirm the aforementioned results that HCMV gB has no effect on the growth of MDA-MB-231 cells, cell extracts were obtained from 231-NC and 231-GB-OE cells and the protein levels of caspase-3, caspase-9, Bax and Bcl-2, which serve important roles in cell apoptosis, were determined. The results demonstrate that the levels of these proteins were similar in 231-NC and 231-GB-OE cells (Fig. 5), which is consistent with the results from the apoptosis and proliferation assays.

The molecular mechanisms of how HCMV gB inhibits MDA-MB-231 migration were then investigated. As TGF- β serves a prominent role in breast cancer progression and tumor metastasis, TGF- β protein expression was measured in 231-NC and 231-GB-OE cells, and the results indicated that TGF- β expression was significantly inhibited in 231-GB-OE cells compared with the 231-NC cells (Fig. 5A and B). This suggests that HCMV gB may suppress TGF- β expression.

Effect of HCMV gB on Smad2/3. The majority of the biological functions of TGF- β are attributed to a canonical signaling pathway mediated by Smad transcription factors (32). To additionally validate our assumption that the migration may be inhibited via impaired TGF- β /Smad signaling, the phosphorylation and total protein levels of Smad2/3 were measured. As expected, the levels of phosphorylation of Smad2 and Smad3 were decreased in 231-GB-OE cells, compared with the 231-NC cells, while no difference in total Smad2/3 levels was observed (Fig. 6A and B). These data indicated that HCMV gB inhibited TGF- β /Smad signaling. Therefore, it was firstly

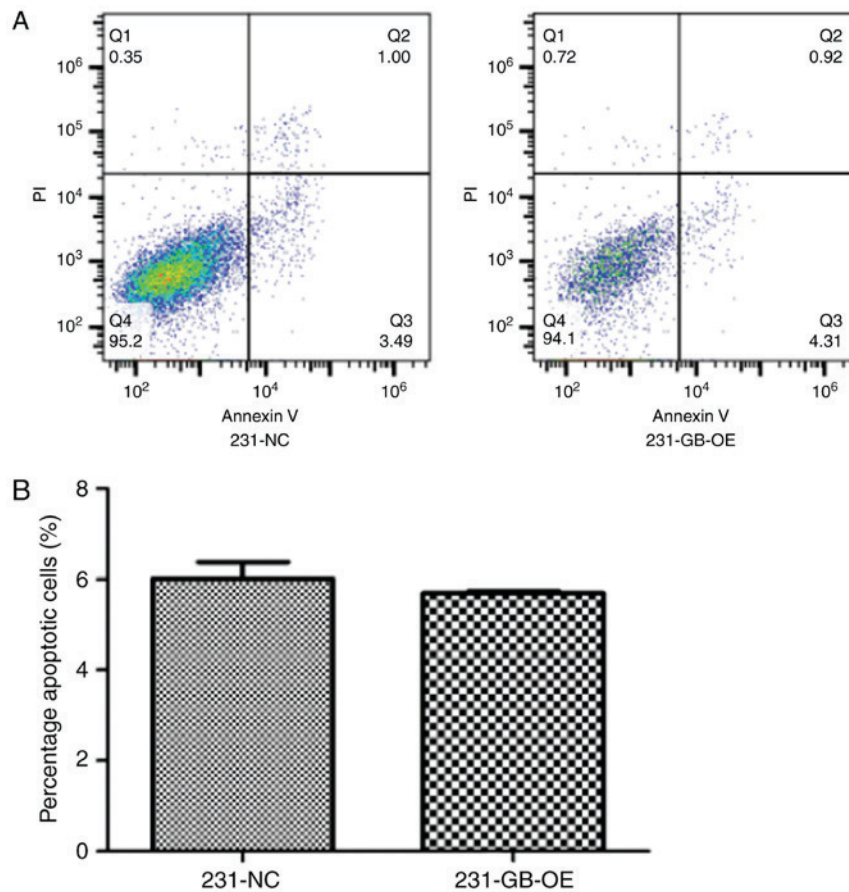


Figure 2. HCMV gB does not demonstrate cytotoxicity in MDA-MB-231 cells. (A) Cell apoptosis was assessed by PI-Annexin V double stain. (B) The percentage of apoptotic cells was quantified. Data are expressed as mean \pm standard deviation. PI, propidium iodide; HCMV, human cytomegalovirus; gB, glycoprotein B.

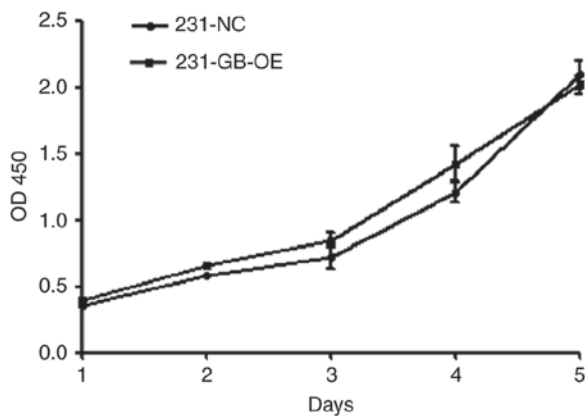


Figure 3. HCMV gB exhibits no effect on cell proliferation. No significant difference in cell proliferation in 231-GB-OE cells compared with the 231-NC cells was observed in the Cell Counting Kit-8 cell proliferation assay. HCMV, human cytomegalovirus; OD, optical density; NC, negative control; gB, glycoprotein B.

suggested that the inhibition of migration may be due to impaired TGF- β /Smad signaling.

Discussion

Although HCMV is not generally regarded as an oncogenic virus, multiple previous studies have demonstrated that HCMV

infection and expression may be specifically associated with certain types of human cancer, including glioblastoma, colon cancer and breast cancer (8,33,34). The HCMV gB was highly expressed during the entire replication cycle of the virus, and responsible for activation of HCMV-induced cellular signaling (35). At present, in order to investigate the effect of gB in glioma cells, Cobbs *et al* (19) introduced HCMV gB into the retroviral vector and transfected into glioma cells, demonstrating the functional expression of HCMV gB by gene sequencing and Western blot analysis: They identified that HCMV gB binds to the Platelet-derived growth factor subunit A (PDGFR α) receptor in glioma cells, mediates entry of HCMV, induces sustained PDGFR α phosphorylation and enhances the invasion of primary glioblastoma cells into Matrigel[®] and rat brain slices. However, an additional study indicated that PDGFR α did not serve as a receptor for HCMV, but increased PDGFR α facilitates virus entry into cells via a novel entry pathway involving clathrin-independent, dynamin-dependent endocytosis of HCMV followed by low pH-independent fusion (36). These studies were performed in glioma cells, and how HCMV gB entry in breast cancer cells remains unclear. To identify whether HCMV gB protein was involved in the effect of HCMV on breast cancer cells, a HCMV gB-expressing 231-GB-OE cell line was established (Fig. 1). Although HCMV has been detected in patients with breast cancer (37) and HCMV gene products was demonstrated to serve in an oncomodulatory manner through the blockage

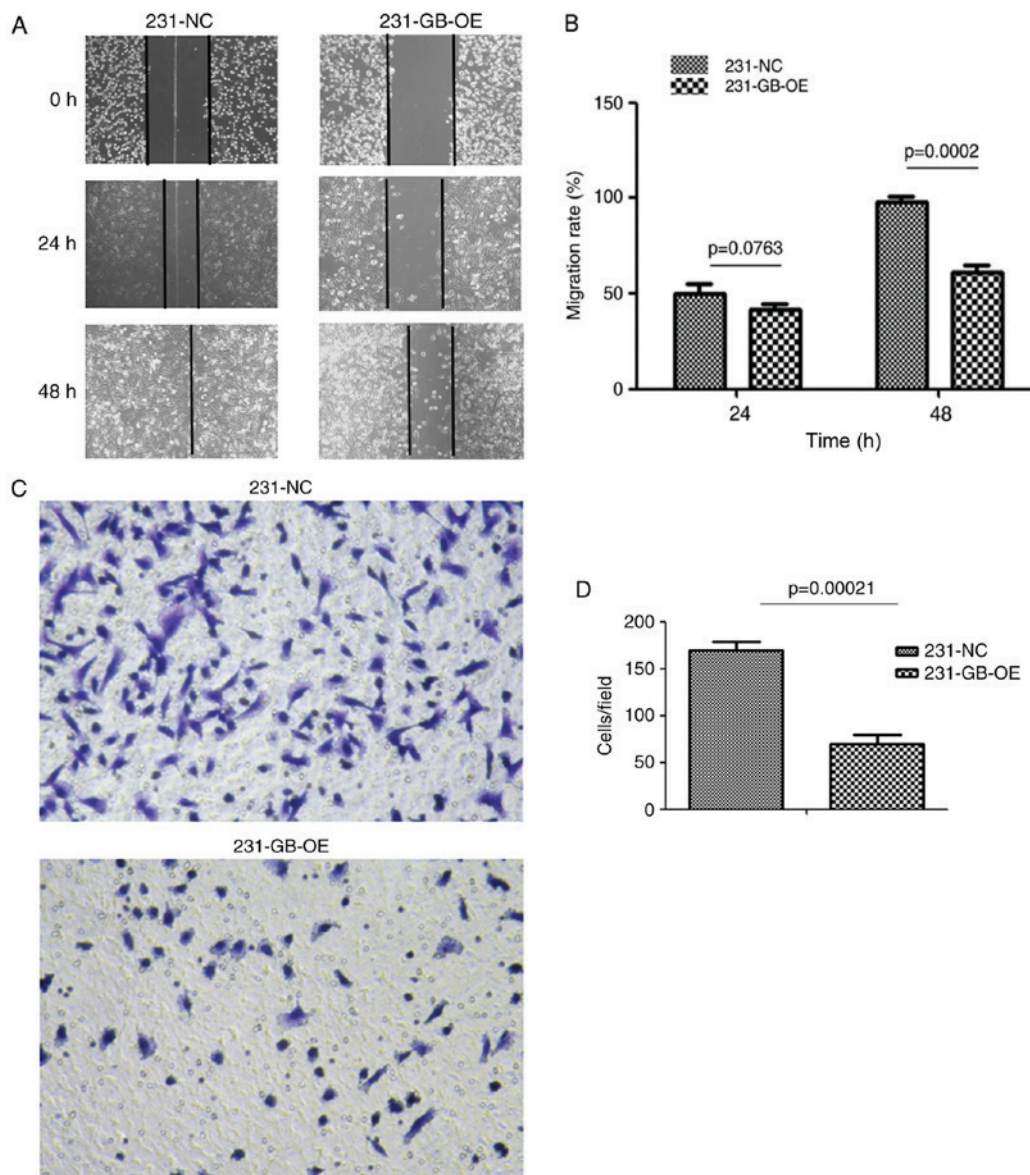


Figure 4. HCMV gB inhibits the migration of 231-GB-OE cells. (A) Wound healing rate in HCMV gB-transfected 231-GB-OE cells was decreased compared with the 231-NC cells. (B) The migration rate from the cell scratch test was quantified. (C) Migrated cells in the lower part following 48 h incubation (x40 magnification). (D) The number of cells in each field under the microscope was quantified. Data are expressed as mean \pm standard deviation. HCMV, human cytomegalovirus; NC, negative control; gB, glycoprotein B.

of cellular differentiation and induction of chromosomal instability (38), the present study indicated that cell viability and proliferation were not affected by HCMV gB (Figs. 2 and 3). Soroceanu and Cobbs (38) demonstrated that HCMV gene products may induce cell migration and angiogenesis; however, the present study identified that HCMV gB inhibited the migration capacity of MDA-MB-231 cells by Transwell assay and by cell scratch test (Fig. 4).

A previous study indicated that TGF- β was a major effector of breast tumor metastasis *in vivo*, and that the inhibition of TGF- β signaling resulted in decreased metastasis in mammary tumors (39). Furthermore, decreased TGF- β responsiveness had no effect on the primary tumorigenesis but significantly decreased cell metastasis in a high-grade breast tumor cell line (40). Consistent with these previous data, the results of the present study suggest that TGF- β downregulation occurred in 231-GB-OE cells and accordingly, the migration capability of

231-GB-OE cells was impaired (Fig. 5). Fynan and Reiss (41) demonstrated that the proliferation of breast tumor cells derived from early stages of the disease was inhibited by TGF- β , while a malignant breast carcinoma cell line exhibited resistance to the anti-proliferative effect of TGF- β . This is consistent with the data from the present study demonstrating that HCMV gB may inhibit MDA-MB-231 migration and TGF- β expression, whilst exhibiting no effect on cell proliferation.

It has been established that the effects of TGF- β are mediated primarily by the receptor-specific Smad2 and Smad3 proteins (42) and active Smad3 signaling contributes to breast cancer local invasion and distant metastasis (43). In concordance with these previous data, the present study identified that the phosphorylation levels of Smad2 and Smad3 were decreased in 231-GB-OE cells compared with 231-NC cells (Fig. 6), indicating that the impaired migration capacity in 231-GB-OE cells may be associated with the inhibited TGF- β /Smad signaling.

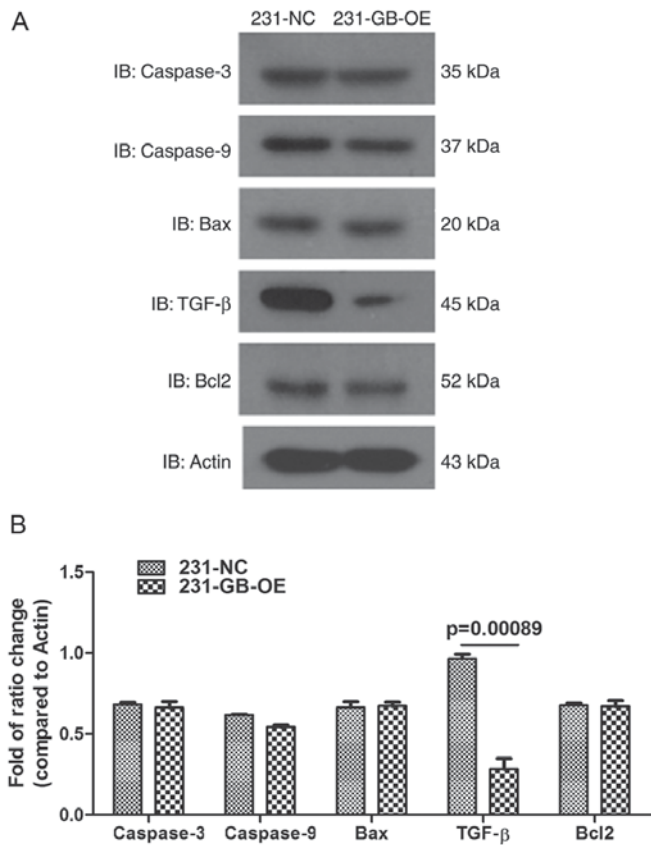


Figure 5. Effect of HCMV gB on caspase-3, caspase-9, Bax and TGF-β expression. (A) Apoptosis-associated protein caspase-3, caspase-9, Bax and Bcl2 were not altered by HCMV gB transfection, while TGF-β was inhibited by HCMV gB. (B) Histogram shows the means of three independent experiments (n=3; mean; SE). HCMV, human cytomegalovirus; NC, negative control; gB, glycoprotein B; IB, immunoblotting.

It was demonstrated previously that in MCF-10-derived breast tumor cells, blockade of TGF-β signal transduction resulted in a decreased chance of the development of metastasis in advanced cancer types (40). Additionally, silencing TGF-β receptor type-I may lead to a reduction in metastasis and invasiveness (44). These implicate TGF-β as an important target for the therapy of advanced cancers and suggest that HCMV gB, which may inhibit TGF-β and Smad2/3, may serve as a promising agent.

In the present study, it was initially demonstrated that although HCMV infection is a risk factor or associated with the development of breast cancer, HCMV gB may inhibit breast cancer cell migration. The expression of TGF-β and phosphorylation of Smad were also downregulated, which is consistent with a number of studies that have demonstrated that TGF-β serves a crucial role in breast cancer migration and metastasis (45-47). However, the present study was not able to clarify how HCMV gB downregulated the phosphorylation of TGF-β and Smad, and future studies will be required to determine whether other signaling pathways are involved and how HCMV gB led to TGF-β/Smad signaling inhibition. In addition, the absence of a gB glycosylation assay to detect protein glycosylation is a limitation of the present study. Burke and Heldwein (48) revealed that 17/18 N-linked glycosylation sites are completely conserved, and hypothesized

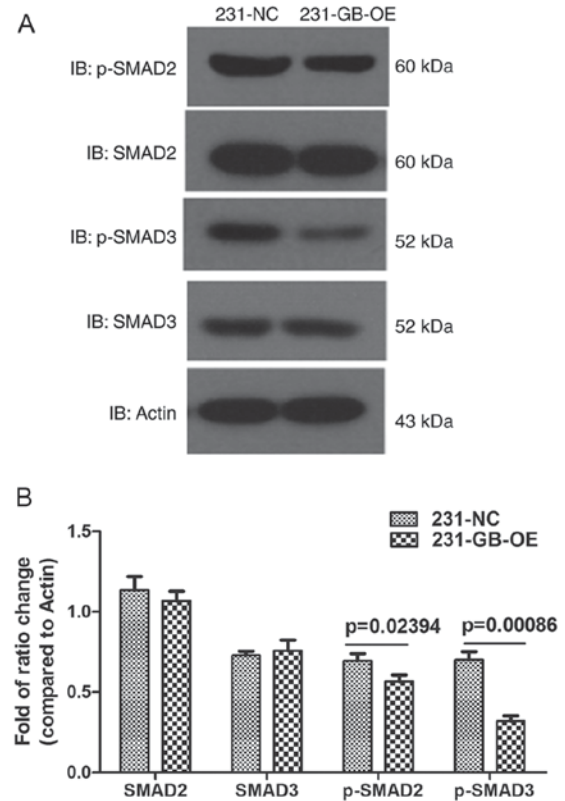


Figure 6. Effect of HCMV gB on activation of Smad2 and Smad3. (A) Total Smad2/3 expression was not changed by HCMV gB while the phosphorylation of Smad2/3 was inhibited. (B) Histogram shows the means of three independent experiments (n=3; mean; SE). HCMV, human cytomegalovirus; NC, negative control; gB, glycoprotein B; Smad2/3, mothers against decapentaplegic 2/3; p-phosphorylated; IB, immunoblotting.

that this high conservation of the glycosylation pattern in HCMV gB suggests that extensive glycosylation in HCMV gB may instead protect functionally important regions from immune recognition, but there are no clear conclusions how glycosylation affects the protein functionality. Taken together, these studies suggest that the identification of HCMV gB as a TGF-β/Smad signal inhibitor may assist in exploitation of the effective therapeutic strategies of the anti-TGF-β agents that contribute to tumor suppression.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article

Authors' contributions

YCL, conceived and designed the experiments; RY, performed the experiments and analyzed the data; JL, manuscript and data discussion; GXX, provided technical guidance during experiments; LMD, HMH, QZS and JY, assisted with performing the experiments.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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