

Human giant larvae-1 promotes migration and invasion of malignant glioma cells by regulating N-cadherin

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Received August 3, 2020; Accepted December 1, 2020

DOI: 10.3892/ol.2021.12428

Abstract. Human giant larvae-1 (Hugl-1) is a human homologue of *Drosophila* tumor suppressor lethal (2)-giant larvae and has been reported to be involved in the development of human malignancies. Previous studies performed by our group demonstrated that Hugl-1 inhibits glioma cell proliferation in an intracranial model of nude mice. However, the exact molecular mechanisms underlying the participation of Hugl-1 in glioma invasion and migration, and in the depolarizing process remain largely unknown. Utilizing the U251-MG cells with stable expression of Hugl-1, the present study used wound healing, Transwell invasion and western blot assays to explore the role and specific mechanism of Hugl-1 in glioma invasion and migration. The results of the present study demonstrated that overexpression of Hugl-1 decreased cell-cell adhesion and increased cell-cell extracellular matrix adhesion. In addition, overexpression of Hugl-1 promoted pseudopodia formation, glioma cell migration and invasion. The molecular mechanism of action involved the negative regulation of N-cadherin protein levels by Hugl-1. Overexpression or knockdown of N-cadherin partially suppressed or enhanced the effects of Hugl-1 on glioma cell migration and invasion, respectively. Furthermore, Hugl-1 inhibited cell proliferation, while promoting cell migration, which suggests that it may serve a two-sided biological role in cellular processes. Taken together, these results suggest that Hugl-1 promotes the migration and invasion of malignant glioma cells by decreasing N-cadherin expression. Thus, Hugl-1 may be applied in the development of targeted and personalized treatment.

Introduction

Glioblastoma (GBM) is the most common malignant primary central nervous system tumor, with an average survival time of 12-15 months (1). The failure of conventional treatments is attributed to its highly invasive and diffusely infiltrative nature (2). Thus, the identification of novel therapeutic targets and strategies to improve the efficacy of existing forms of treatment is urgently required.

Cell polarity and intercellular adhesion play a key role in regulating normal tissue structure and function (3). The disruption of cell polarity and cell adhesion is usually associated with tumor formation (4). Lethal (2)-giant larvae (Lgl) is a cortical cytoskeletal protein, which was initially identified in *Drosophila* and exhibits notable effects in the establishment and maintenance of apical-basal epithelial polarity, asymmetric cell division, tissue integrity and cell proliferation (5). The human homologues of Lgl1 and Lgl2 are termed human giant larvae (Hugl)-1 and Hugl-2. Mutations that cause loss of function of Lgl have been demonstrated to result in tissue overgrowth and neoplastic tumor formation (6,7). The Hugl-1 protein shares 62.5% similarity with Lgl (8-10). A previous study indicated that hepatocellular carcinoma (HCC) contains frequent mutations of Hugl-1, whereas overexpression of HCC-derived aberrant Hugl-1 variants significantly promote HCC cell migration and invasion (11). In addition, Hugl-1 expression is downregulated in different types of human cancer, including colorectal cancer, melanoma, prostate cancer, breast cancer, endometrial cancer, lung cancer and esophageal carcinoma (12-15). Hugl-1 expression is positively associated with a higher survival rate in patients with pancreatic carcinoma, suggesting its use as a reliable prognostic marker (16). The majority of previous studies have focused on epithelial-derived tumors (11-15), thus the role of Hugl-1 in gliomas (glia-derived tumors) has not yet been fully elucidated. A previous study performed by our group has demonstrated that Hugl-1 protein levels decrease in human glioma tissues, whereas overexpression of Hugl-1 attenuates glioma cell proliferation in an intracranial model of nude mice; however, it does not affect glioma cell proliferation *in vitro* (17). As a regulator of cell polarity, Hugl-1 exhibits important properties that are closely associated with cell adhesion and cytoskeletal function and structure (18). However, the role of Hugl-1 in glioma migration and invasion has not yet been fully investigated.

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Key words: glioma, Hugl-1, migration, invasion, N-cadherin

Cell surface adhesion molecules are the main mediators of cell-cell interactions, which are essential for tumor malignant biological behaviors. Reorganization of the cell cytoskeleton and alteration of cell-cell adhesion are required prior to cell migration (19,20). These processes are mainly mediated by cadherin family members. It is reported that E-cadherin is essential for the normal migration of cranial neural crest cells *in vivo*, while P-cadherin, also known as placental cadherin, is associated with malignant invasion of esophageal squamous cells (21-24). In most tumors, N-cadherin expression is often upregulated and can be used as a promoter of tumor invasion (25,26). N-cadherin expression in epithelial cells can induce morphological changes of fibroblast phenotype and orchestrate cell-cell communication during cell movement (27). N-cadherin is also known as an epithelial-to-mesenchymal transition marker and exhibits several functions according to the cell environment that can promote adhesion or induce migration (28,29). However, increasing evidence suggests that N-cadherin exhibits tumor-inhibitory roles in non-epithelial derived neoplasms, such as osteosarcoma and glioma (27,30). Thus, the functions of N-cadherin may be tumor-type specific (27).

The present study aimed to investigate the role and molecular mechanism of HUGL-1 on the motility of malignant glioma cells.

Materials and methods

Cell culture. The U251-MG glioma cell line was purchased from the Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences. Cells were maintained in DMEM/F-12 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Biological Industries), at 37°C in 5% CO₂.

Stable transfection of HUGL-1 into U251-MG cells. The pEGFP-C1 vector alone or the pEGFP-C1-HUGL-1 construct (provided by Professor Zhengjun Chen, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) was transfected into U251-MG cells (GFP-Vector or GFP-HUGL-1 cells, respectively) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, 9 µl Lipofectamine[®] 2000 and 3 µg of the HUGL-1 expression plasmid were added to 1 ml Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 10 min at room temperature. The plasmid and Lipofectamine were mixed together and incubated for 30 min before adding them to the U251-MG cells. The transfectants were subsequently selected using G418 (1,200 µg/ml), and single-cell clones were obtained following 3-4 weeks of growth for expansion. The G418-resistant cells were used for subsequent experiments. DsRed-C1 or DsRed-N-cadherin plasmids were kindly provided by the Laboratory of Cell Biology of Northeast Normal University (Changchun, China). DsRed-C1 or DsRed-N-cadherin plasmids were transfected into HUGL-1 overexpressing U251-MG cells. The specific transfection procedure was the same as that of HUGL-1.

Digestion assay. Cultured GFP-Vector or GFP-HUGL-1 cells were digested with trypsin simultaneously (Gibco; Thermo Fisher Scientific, Inc.). Briefly, cells were digested with trypsin

at room temperature for 8 min and observed at designated time points (0, 2, 4 and 8 min) under an inverted light microscope during trypsinization at x200 magnification (Olympus Corporation; IX71).

Attachment assay. The attachment assay was performed using 12-well plates. The cell suspension was added into the plates and cell images were obtained at 3, 6, 9 and 24 h using an inverted light microscope at x400 magnification (Olympus Corporation; IX71).

Wound healing assay. Cell migration was assessed via the wound healing assay, as previously described (31). Briefly, cells were seeded into 6-well plates and cultured until they reached ~80% confluence. The cell monolayers were scratched using a 10 µl sterile pipette tip. Cells were subsequently washed twice with PBS to remove floating cells and serum-free DMEM/F-12 medium (Gibco; Thermo Fisher Scientific, Inc.) was added. Cell wound healing was observed at 0, 24 or 48 h using an inverted light microscope at x200 magnification (Olympus Corporation; IX71).

Transwell invasion assay. The cell invasion assay was performed as previously described (31). Briefly, Transwell membranes were precoated with DMEM-diluted Matrigel[®] (BD Biosciences) for 3 h at 37°C. Cells (2x10⁴) were plated in the upper chambers of Transwell plates in 200 µl serum-free culture DMEM/F-12 medium (Gibco; Thermo Fisher Scientific, Inc.). A total of 500 µl DMEM/F-12 medium supplemented with 10% FBS was plated in the lower chambers. Following incubation at room temperature for 24 h, the invasive cells were fixed in methanol for 15 min at room temperature and subsequently stained for 15 min at room temperature with 0.1% crystal violet. Invasive cells were viewed and counted under an inverted light microscope at x200 magnification (Olympus Corporation; IX71).

Western blotting. U251-MG cells were lysed with RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktail, and total proteins were quantified using a BCA kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Western blotting was performed as previously described (32). Briefly, equal amounts of protein (20 µg/lane) were separated by SDS-PAGE on 8 or 10% gels, transferred onto polyvinylidene fluoride membranes and blocked using 3% BSA (Sangon Biotech Co., Ltd.) for 2 h at room temperature. The membranes were then incubated with primary antibodies against HUGL-1 (1:500) [kindly gifted by Dr ZG Luo from the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (33)], N-cadherin (1:2,000; cat. no. ab76011; Abcam), β-catenin (1:5,000; cat. no. ab32572; Abcam), integrinβ1 (1:1,000; cat. no. ab134179; Abcam) and β-actin (1:1,000; cat. no. MABT523; EMD Millipore). Following the primary antibody incubation at 4°C overnight, membranes were probed with HRP-labelled goat anti-rabbit or anti-mouse IgG secondary antibodies (1:4,000; cat. nos. sc2004 and sc2005; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The signal was detected using the Pierce ECL Plus Western

Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) and exposed to ChemiDoc Touch (Bio-Rad Laboratories, Inc.). Finally, gray analysis was performed using ImageJ 1.48V (National Institutes of Health) to compare the level of each protein.

Phalloidin staining. U251-MG cells were incubated for 24 h at 37°C and cultured in DMEM/F-12 medium supplemented with 10% FBS for 30 min at 37°C. Subsequently, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and 0.5% Triton X-100 was added for 5 min at room temperature. Finally, 200 μ l of the diluted phalloidin (cat. no. 94072; Sigma-Aldrich; Merck KGaA) was added and incubated at room temperature in the dark for 30 min. Actin filaments were observed using an inverted fluorescence microscope at x400 magnification (Olympus Corporation; IX71).

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc.). All experiments were performed in triplicate and data are presented as the mean \pm standard error of the mean. Student's unpaired t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of HUGL-1 on cell adhesive activity. It has been demonstrated that HUGL-1 protein expression is downregulated in human glioma tissues compared with normal brain tissues (17). Given that HUGL-1 acts as a tumor suppressor in several human tumors, and its expressed at low levels in gliomas, the effect of HUGL-1 downregulation on this basis may be negligible. In addition, we detected six different glioma cell lines in the previous study and demonstrated that HUGL-1 protein expression was extremely low in U251-MG cells (17). Thus, to investigate the role of HUGL-1 in glioma, stable GFP-HUGL-1 overexpression was established in U251-MG glioma cells, and constitutive expression was assessed via western blotting (Fig. 1A and B). Due to the high molecular weight of HUGL-1 (115 kDa) (13), the molecular weight of GFP-HUGL-1 fusing protein was 141 kDa (115 kDa + 26 kDa), which caused weaker GFP signaling in the GFP-HUGL-1 group compared with the GFP group. To determine the difference in the adhesive ability between the GFP-HUGL-1 and GFP groups, cells were trypsinized, and the results demonstrated that HUGL-1 overexpressing cells retracted more slowly compared with the GFP control cells (Fig. 1C), suggesting a better cell-extracellular matrix adhesive ability. Conversely, HUGL-1 overexpressing cells extended pseudopodia faster than GFP cells following plating (Fig. 1D). Notably, GFP cells formed cell aggregates unlike HUGL-1 overexpressing cells (Fig. 1D, black arrow), suggesting that upregulation of HUGL-1 decreases cell-cell adhesive activity. The cells presented in Fig. 1D exhibited complete roundness and good refraction, indicating that the cells were healthy. In addition, the digested cells were placed in a culture dish and gradually expanded.

N-cadherin is a member of the calcium-dependent adhesion molecule family, which mediates adhesion between homotypic cells (34). Thus, N-cadherin protein expression was detected at 3, 6 and 24 h following plating. The results demonstrated that N-cadherin expression was lower in HUGL-1 overexpressing cells compared with GFP cells at 3 and 6 h following plating (Fig. 1E and F).

Previous studies have demonstrated that adhesion molecules play an important role in the early stage of cell adhesion (35,36), which gradually decreases overtime (37). In the present study, no significant difference was observed in N-cadherin expression between the two groups 24 h after plating. Taken together, these results suggest that overexpression of HUGL-1 decreases cell-cell adhesion, while increasing cell-extracellular matrix adhesion by regulating N-cadherin expression.

HUGL-1 accelerates cytoskeletal remodeling. To fully characterize the intercellular adhesion defects observed in HUGL-1 overexpressing cells, the intracellular organization of the cytoskeleton was assessed. Cells were incubated for 24 h and cultured in media supplemented with 10% FBS for 30 min. Subsequently, cells were stained with phalloidin-conjugated actin to assess actin reassembling. The results demonstrated that HUGL-1 overexpressing cells expanded their lamellipodia earlier, which contained concentrated F-actin, and stretched faster than GFP cells (Fig. 2A). Previous studies have established β -catenin as a promoter signal, which is not only a key transcription factor in the Wnt signaling pathway but also a structural adaptor between cadherin and actin skeleton during cell adhesion (38,39). The present study hypothesized that β -catenin may mediate cytoskeleton remodeling by overexpressing HUGL-1. The results of the present study demonstrated that β -catenin expression decreased in HUGL-1 overexpressing cells, while the expression levels of integrin β 1, another important molecule involved in cytoskeleton remodeling and adhesion (40), remained unchanged (Fig. 2B and C). Collectively, these results suggest that HUGL-1 may accelerate cell cytoskeleton reorganization by regulating the N-cadherin- β -catenin complex.

HUGL-1 promotes the migration and invasion of glioma cells. The effect of overexpressing HUGL-1 on the migration of glioma cells was assessed via the wound healing assay. The results demonstrated that HUGL-1 overexpressing glioma cells exhibited faster wound healing than GFP cells after 48 h (Fig. 3A and B), whereby the number of migratory cells significantly increased to $30 \pm 7\%$ ($P < 0.01$; Fig. 3A and B). In addition, the effect of overexpressing HUGL-1 on the invasion of glioma cells was assessed via the Transwell assay. The results demonstrated that the number of invasive cells significantly increased to $139 \pm 5\%$ following overexpression of HUGL-1 ($P < 0.01$; Fig. 3C and D), which confirms that HUGL-1 promotes the invasive ability of U251-MG cells.

N-cadherin partially mediates the effects of HUGL-1 expression on glioma cell migration. N-cadherin plays a key role in regulating cell polarity and motility (27). Based on the results presented in Fig. 1E, whether N-cadherin mediates the effects of HUGL-1 expression on glioma cell migration

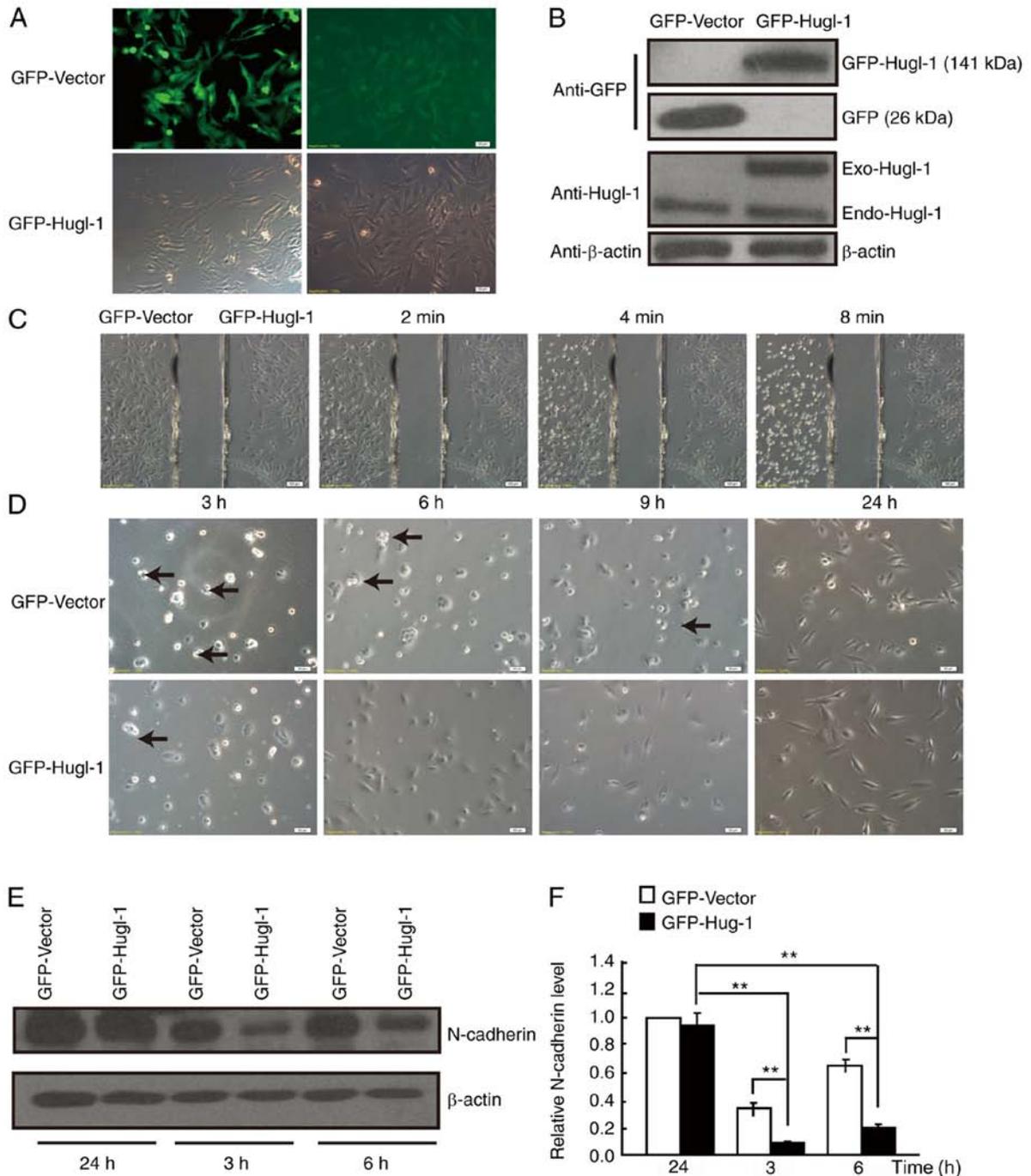


Figure 1. Hugl-1 affects cell adhesion. (A) GFP-Hugl-1 or GFP-Vector plasmids were transfected into U251-MG glioma cells, followed by G418 selection. The transfection efficiency was assessed via GFP fluorescence (scale bar, 50 μm). (B) Western blot analysis was performed to detect Hugl-1 protein expression. (C) Representative digital images obtained at 0, 2, 4 and 8 min during trypsinization (scale bar, 100 μm). (D) Representative digital images obtained at 3, 6, 9 and 24 h following plating. Black arrowheads indicate the cell aggregates (scale bar, 50 μm). (E) N-cadherin protein levels were detected at 3, 6 and 24 h following cell attachment. (F) Quantification results of (E). **P<0.01. Hugl-1, human giant larvae-1; GFP, green fluorescent protein.

was subsequently assessed. N-cadherin was overexpressed in GFP and Hugl-1 overexpressing cells (Fig. 4A), and the results demonstrated that upregulation of Hugl-1 promoted cell migration, the effects of which were reversed following overexpression of N-cadherin (Fig. 4B and E).

Generally, the exogenous protein level is higher than that of the endogenous level. However, considering the high molecular weight of GFP-Hugl-1 (141 kDa) and N-cadherin (127 kDa), and the relatively low transit transfection efficiency (12,13), the exogenous protein level was lower than the endogenous level

in the present study (Fig. 4A). Notably, the induction effect of Hugl-1 on glioma cell migration was partially abolished following overexpression of N-cadherin, compared with that of the Hugl-1 overexpressing cells (Fig. 4B and E). Conversely, N-cadherin knockdown promoted cell migration, and the effects were similar to those noted in the Hugl-1 overexpressing cells (Fig. 4C, D and F). Furthermore, the increased migratory ability induced by Hugl-1 upregulation was significantly enhanced following downregulation of N-cadherin in glioma cells (Fig. 4D and F). Taken together, these results suggest that

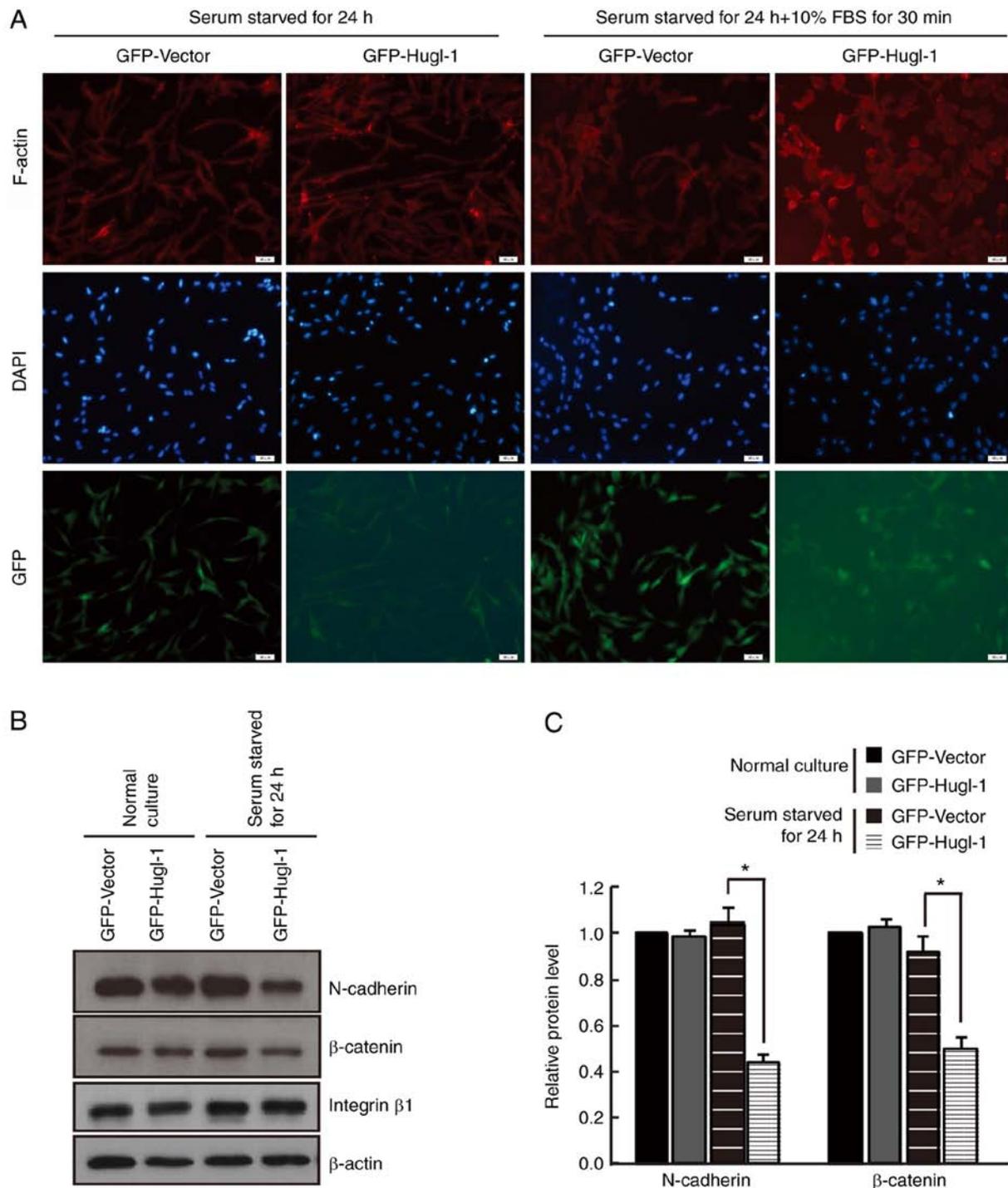


Figure 2. Hugl-1 accelerates cytoskeletal remodeling. (A) Following serum starvation for 24 h, GFP and Hugl-1 overexpressing cells were stimulated with 10% FBS for 30 min and stained with conjugated phalloidin (red). The images indicated that more dot or fan-like protrusions were detected at the cell periphery in Hugl-1 overexpressing cells. (B) Western blot analysis was performed to detect the protein expression levels of N-cadherin, β-catenin and integrin β1 in GFP and Hugl-1 overexpressing cells. (C) Quantification results of (B). Scale bar, 50 μm. *P<0.05. Hugl-1, human giant larvae-1; GFP, green fluorescent protein; FBS, fetal bovine serum.

N-cadherin partially mediates the effects of Hugl-1 expression on glioma cell migration.

Discussion

Intercellular adhesion plays a crucial role in the maintenance of cell polarity to regulate normal tissue architecture and function (41,42). This process is often disrupted in neoplastic

tumors (43,44). Loss of polarity is considered one of the trigger signals for tumorigenesis and invasion of surrounding tissues (45,46). As a cell polarity regulator, Hugl-1 expression is downregulated in several types of human cancer, such as squamous cell lung carcinoma (15), esophageal carcinoma (47), pancreatic carcinoma (16), endometrial cancer (14) and malignant melanoma (12), and is inversely associated with patient prognosis (11-15). We previously demonstrated that Hugl-1 can

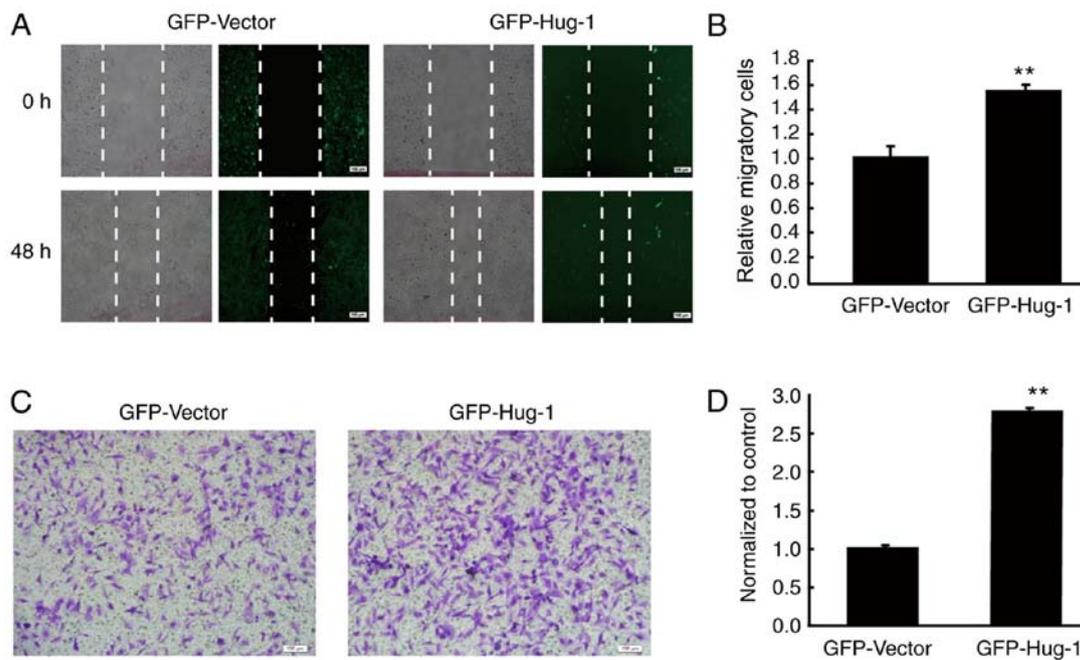


Figure 3. Hugl-1 promotes migration and invasion of glioma cells. (A) Representative digital images of the wound healing assay taken at 0 and 48 h following scratching. (B) Quantification results of (A). (C) The invasive ability of glioma cells was assessed via the Transwell assay. (D) Quantification results of (C). Scale bar, 100 μ m. ** $P < 0.01$ vs. GFP-Vector. Hugl-1, human giant larvae-1; GFP, green fluorescent protein.

inhibit tumor progression *in vivo*, while no significant effects on cell proliferation were observed *in vitro* (17).

The results of the present study demonstrated that overexpression of Hugl-1 decreased cell-cell adhesion, probably by regulating N-cadherin protein expression. In addition, overexpression of Hugl-1 promoted glioma cell migration and invasion. Notably, overexpression or knockdown of N-cadherin partially suppressed or enhanced the induction effect of Hugl-1 expression on glioma cell migration and invasion, respectively. Taken together, these results suggest that Hugl-1 promotes migration and invasion of malignant glioma cells by decreasing N-cadherin expression, thus Hugl-1 may act as a novel therapeutic target in patients with GBM, and function as a marker of GBM prognosis.

Schimanski *et al* (13) demonstrated that Hugl-1 expression is lost in 75% of tumor samples and that these deletions are associated with advanced disease stage, particularly with lymph node metastasis. Similarly, loss of Hugl-1 expression in endometrial cancer may contribute to lymph node metastasis (14). Notably, overexpression of wild-type Hugl-1 inhibits HCC migration and invasion (11). Kuphal *et al* (12) reported that upregulation of Hugl-1 increases cell adhesion and decreases cell migration in malignant melanoma. However, the results of the present study demonstrated that overexpression of Hugl-1 promoted glioma cell migration and invasion. Although Hugl-1 expression decreases in malignant melanoma, HCC and gliomas, it exhibits opposite effects on cell migration and invasion (promotion versus inhibition) in different types of tumors (11,12,17). These differences may be due to the different cell types used in each experiment under specific conditions. Kuphal *et al* (12) and Lu *et al* (11) used Mel Im or SK-HEP-1 cells, which are epithelial cells, while the present study used U251-MG glioma cells, a cell type that belongs to glia-derived

cells (48). However, whether the functions of Hugl-1 are cell-type specific remains unknown and should be investigated in prospective studies.

Cell migration and invasion include multiple processes, such as extracellular matrix degradation, cytoskeletal reorganization, de-adhesion and adhesion (49,50). Cytoskeletal reorganization is an important process that affects assembly and disassembly of cell-cell adhesions and leads to morphological and motility changes of tumor cells (50). It is well-known that low expression levels of Hugl-1 in gliomas decrease cell-cell adhesion, promote cell migration and ultimately contribute to cancer cell dissemination and tumor progression (51). However, the results of the present study are not consistent with these conclusions, suggesting that the balance of polar proteins may be the optimum condition for maintaining cell homeostasis (52). In addition, the results of the present study demonstrated that overexpression of Hugl-1 significantly promoted pseudopodia formation and supported the enhanced cell-extracellular matrix adhesion. Asano *et al* (53) reported that N-cadherin expression is negatively associated with tumor invasion. The results of the present study demonstrated that overexpression of Hugl-1 decreased cell-cell adhesion and increased cell migration, which was consistent with the decreased protein levels of N-cadherin. Recently, Jossin *et al* (54) reported that LLGL1 directly binds to N-cadherin and is able to promote its internalization, while disrupting the N-cadherin-LLGL1 interaction, which results in cortical heterotopias. The results of the present study are consistent with these findings.

In conclusion, the results of the present study demonstrated that Hugl-1 promoted glioma cell migration and invasion by decreasing N-cadherin expression. Combined with our previous studies, the results presented here provide a novel role for Hugl-1, which includes inhibition of cell proliferation, while promoting cell migration in glioma, suggesting that Hugl-1

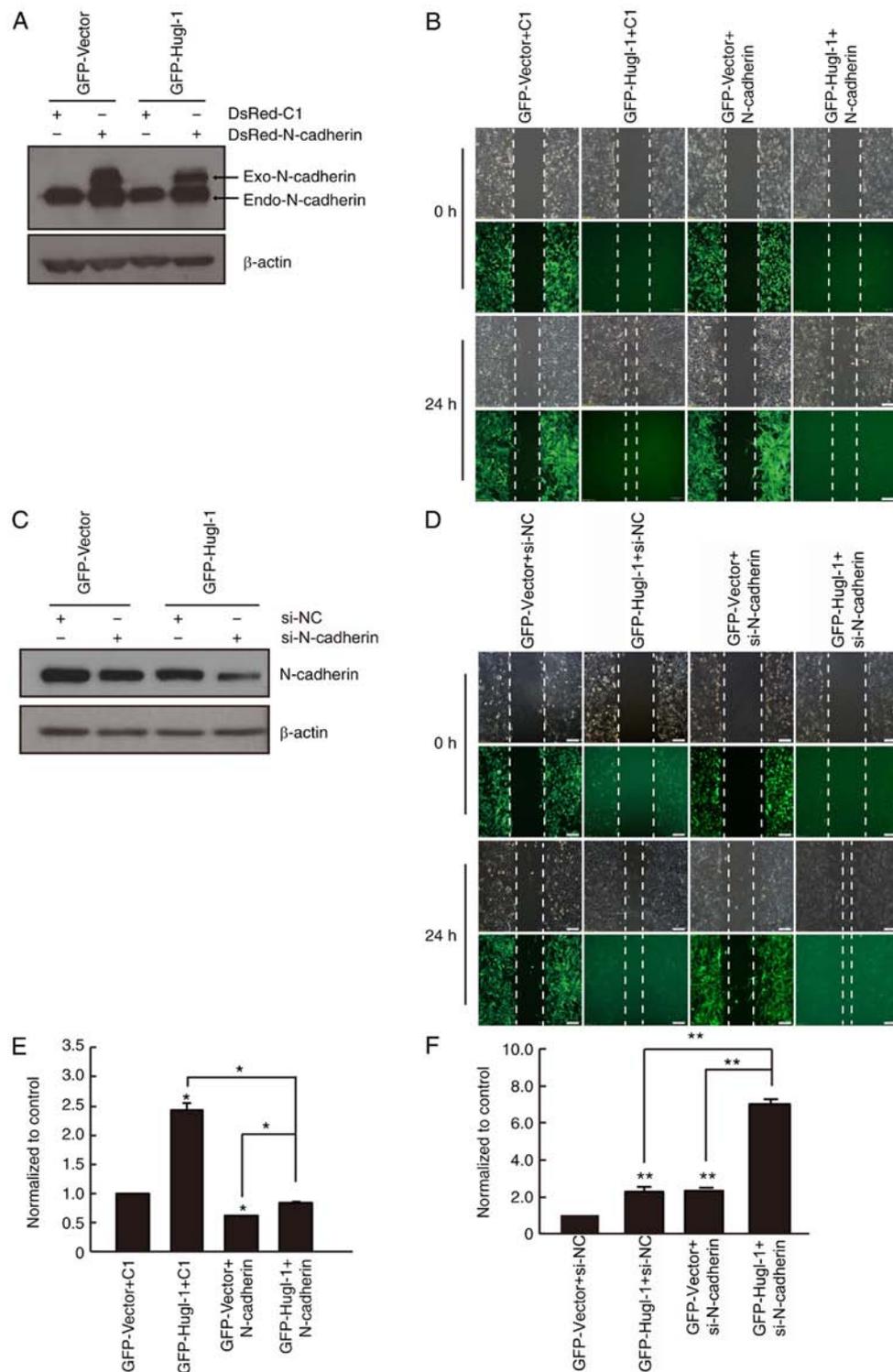


Figure 4. N-cadherin partially mediates the effect of Hugl-1 expression on glioma cell migration. (A) The overexpression efficiency of N-cadherin in U251-MG cells was detected via western blot analysis. (B) The wound healing assay was performed to assess cell migration. (C) The downregulation efficiency of N-cadherin was detected via western blot analysis. (D) The wound healing assay was performed to assess cell migration. (E) Quantification results of (B). (F) Quantification results of (D). Scale bar, 100 μ m. * P <0.05, ** P <0.01. Hugl-1, human giant larvae-1; GFP, green fluorescent protein; exo, exogenous; endo, endogenous; si, small interfering; NC, negative control.

may play two-sided roles in malignant biological processes. In addition, the results presented here provide useful information for the clinical diagnosis of malignant GBM and the prognosis of patients with GBM. Further studies are required to determine the exact role and precise molecular mechanism of the cell polarity molecule, Hugl-1, for the effective treatment of

glioma. Although the present study investigated the role of Hugl-1 in glioma cells, it still presented some limitations. The current experiments were all completed at the cellular level; therefore, there is a lack of detection in animal experiments, which should be further explored in future studies to confirm the present findings.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 81672489, 81872053 and 81902526) and the Postgraduate Research & Practice Innovation Program of Jiangsu Province (grant no. KYCX20_2460).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW wrote the manuscript and contributed to data analysis. YZ, BS and XZ performed the experiments. RY contributed to the study design. XZ contributed to the study design, reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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