Myosin Heavy Chain 10 (MYH10) Gene Silencing Reduces Cell Migration and Invasion in the Glioma Cell Lines U251, T98G, and SHG44 by Inhibiting the Wnt/β-Catenin Pathway

AB 1 Yang Wang*
AB 2 Qi Yang*
CD 3 Yanli Cheng
DE 4 Meng Gao
CE 5 Lei Kuang
EF 6 Chun Wang

* These authors contributed equally to this work

Corresponding Author: Chun Wang, e-mail: chunw_wangc@163.com

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Background: The myosin heavy chain 10 or MYH10 gene encodes non-muscle myosin II B (NM IIB), and is involved in tumor cell migration, invasion, extracellular matrix (ECM) production, and epithelial-mesenchymal transition (EMT). This study aimed to investigate the effects of the MYH10 gene on normal human glial cells and glioma cell lines in vitro, by gene silencing, and to determine the signaling pathways involved.

Material/Methods: The normal human glial cell line HEB, and the glioma cell lines, U251, T98G, and SHG44 were studied. Plasmid transfection silenced the MYH10 gene. The cell counting kit-8 (CCK-8) assay evaluated cell viability. Cell migration and invasion were evaluated using scratch and transwell assays. Western blot measured the protein expression levels, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the mRNA expression levels, for MYH10, metastasis-associated protein 1 (MTA-1), matrix metalloproteinase (MMP)-1, MMP-9, tissue inhibitor of metalloproteinases 2 (TIMP2), collagen 1, E-cadherin, vimentin, Wnt3a, β-catenin, and cyclin D1.

Results: The MYH10 gene was overexpressed in U251, T98G, and SHG44 cells. MYH10 expression was down-regulated following siMYH10 plasmid interference, which also inhibited glioma cell migration and invasion. MYH10 gene silencing resulted in reduced expression of MTA-1, MMP-2, MMP-9 and vimentin, and increased expression of TIMP-2, E-cadherin and collagen 1 at the protein and mRNA level, and inhibited the Wnt/β-catenin pathway.

Conclusions: In human glioma cell lines, silencing the MYH10 gene reduced cell migration and invasion, by inhibiting the Wnt/β-catenin pathway, which may regulate the ECM and inhibit EMT in human glioma.

MeSH Keywords: Cell Migration Inhibition • Epithelial-Mesenchymal Transition • Glioma • Myosin Heavy Chains • Neoplasm Invasiveness

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Background

The malignant form of glioma, glioblastoma, is the most common primary malignant tumor of the brain, comprising 16% of all primary tumors of the brain and central nervous system (CNS) [1]. It is challenging to treat malignant gliomas, due to their high recurrence rate and low survival rate [2]. Currently, the main methods of treatment include radiotherapy, chemotherapy or surgery [1,3]. However, due to the special location of these tumors, there are limitations in the use of radiotherapy and chemotherapy, which may be associated with side effects that limit the patient’s tolerance to these treatments [4]. Because malignant gliomas are highly invasive and the tumor borders may not be clear, surgical resection is also difficult [5]. Therefore, there are several ongoing studies to study the pathogenesis of malignant glioma, including the mechanisms involved in tumor invasion, and to identify new therapeutic targets.

The myosin heavy chain 10 or MYH10 gene encodes non-muscle myosin II B (NM IIB) and is involved in tumor cell migration, invasion, extracellular matrix (ECM) production, and epithelial-mesenchymal transition (EMT). The MYH10 gene is located on human chromosome 17, and the encoded protein, NM IIB, is mainly present in nerve cells, megakaryocytes, and other non-muscle cells [6,7]. NM IIB has a role in cell adhesion and migration [8]. A previously published study showed that the MYH10 gene was overexpressed in breast cancer and that increased expression of MYH10 was associated with tumor cell invasion in breast cancer [9]. A previously published study also showed that mutations in the MYH10 gene promoted cell migration and were associated with metastasis of malignant tumors [10]. However, there have been no previously published studies on the effect of MYH10 gene expression on the invasive properties of human glioma cells.

Cell migration and invasion are characteristics of tumor cells in vitro [11]. The invasion and migration of tumor cells in vivo is associated with the components of the extracellular matrix (ECM) and the effects of epithelial-mesenchymal transition (EMT) [11]. ECM and EMT are regulated by several signaling pathways [12,13]. Importantly, overexpression of the Wnt/β-catenin pathway has been shown to be associated with metastasis of several types of malignancy [14–16]. RNA interference technology is an effective method to study the function of genes and has demonstrated benefits in the rapid discovery of effective targets of drug action, confirmed by in vitro cell culture and other pre-clinical experimental techniques [17].

Therefore, the aim of this study was to investigate the effects of the MYH10 gene on normal human glial cells and glioma cell lines in vitro, by gene silencing, and to determine the effects on the expression of ECM and EMT-associated proteins and the Wnt/β-catenin pathway related proteins.

Material and Methods

Cells culture

The normal glial cell line, HEB, and the human glioma cell lines, U251, T98G, and SHG44, were purchased from Shanghai Beinuo Biotech Co., Ltd. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) including 10% fetal calf serum, penicillin and streptomycin 100 U/mL (HyClone Laboratories Inc., South Logan, UT, USA) in an incubator at 37°C in 5% CO₂.

MYH10 gene interference plasmid construction and transfection

The MYH10-small interfering RNA (siRNA) plasmid (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with Lipofectamine 2000, and OPTI-MEM™ reduced serum medium (Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 1: 2.5: 250 and allowed to stand for 20 min at room temperature to obtain a liposome mixture. MYH10-negative-siRNA (without the siRNA plasmid) (Thermo Fisher Scientific, Waltham, MA, USA) was used as the normal control (NC) group.

The T98G human glioma cell line was placed in a DMEM medium, cultured at 37°C in 5% CO₂, and seeded into a cell culture plate overnight. The inoculation density was 1×10⁴ cells/mL. After removing of the cell culture medium, cells were added with 100 μL of the MYH10-siRNA-liposome mixture and the MYH10 without the siRNA plasmid liposome mixture respectively, and then cultured for 6 h. DMEM culture medium was added, and further culture was performed for 24 h. The cells were passaged at a ratio of 1: 10, cultured in 1 μg/mL puromycin media (Becton Dickinson, Franklin Lakes, NJ, USA) until a confluent clonal cell population was grown, at which time, cells were selected and seeded in 96-well cell culture plates. When the cells reached 70% confluence, they were transferred to culture flasks. Finally, three experimental groups of cells were obtained for further study: the MYH10-siRNA interference group (the siMYH10 group), the negative-siRNA interference group, without the siRNA plasmid (the NC group), and untransfected group (the NC or control group). The transfection efficiencies were detected using quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot, respectively.

Cell viability using the cell counting kit-8 (CCK-8) assay

The cell counting kit-8 (CCK-8) assay (Tong Ren Tang Technologies Co Ltd, Beijing, China) was used to detect cell viability at 12 h, 24 h, and 48 h after plasmid transfection of the T98G cells. The cells were inoculated onto 96-well plates and pre-incubated at 37°C in 5% CO₂, and then 10 mL of CCK-8 reagent was added, and culture was continued at 37°C in 5% CO₂ for 4 h. The absorbance of each well at 450 nm
was measured by using an ELX 800 microplate reader (BioTek, Winooski, VT, USA), and cell viability was calculated according to the standard curve.

**Scratch assay**

A black marker pen was used to draw a horizontal line across each well of the 6-well plate. Each horizontal line had a fixed interval of 0.5 cm. Then, 5×10⁴ of viable cells were added to each well and incubated overnight at 37°C in 5% CO₂. The fusion rate achieved was 100%. After using the tip of the pipette perpendicular to the black line behind the plate to scratch the culture, the cells were gently washed with PBS buffer and incubated for 24 h in serum-free medium (Thermo Fisher Scientific, Waltham, MA, USA). After taking pictures, ImageJ software was used to process the images and calculate the cell mobility.

**Transwell analysis**

Transwell chamber and related reagents were purchased from Corning (New York, NY, USA). The cells were digested with trypsin for 5 min, and 500 mL of the cell culture was added in the lower chamber, and 5 × 10⁴ cells were added and cultured for 4 h. The cells from the upper chamber were added to the lower chamber. Finally, a cell suspension containing 5×10⁴ cells was added into the upper chamber vertically, dropwise, followed by culture for 6–8 h at 37°C and 5% CO₂. After staining with Giemsa (Shanghai Gefan Biotechnology Co., Ltd., China), five high-power fields were randomly selected using light microscopy to quantify the rate of cell invasion.

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

The cells were titrated and lysed using TRIzol (Takara, Minato-ku, Tokyo, Japan) at 0°C for 5 minutes. The RNAs were extracted using carbon tetrachloride (CCl₄) and dissolved in diethyl pyrocarbonate (DEPC) water (Sigma). RNA concentration was measured using a NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and cell viability was calculated according to the standard curve.

RT-qPCR experiments were performed using the SYBR Premix Ex Taq™ Real-Time PCR Kit (Takara, Minato-ku, Tokyo, Japan). PCR was performed by activating the DNA polymerase at 95°C for 5 min, followed by 40 cycles of a two-step PCR (95°C for 10 sec and 60°C for 30 sec) and a final extension at 75°C for 10 min and held at 4°C. DNase and RNase-free water were used as the templates of negative control experiences. All primers were obtained from Genewiz (Suzhou, Jiangsu, China), and are listed in Table 1. GAPDH was used as an internal control.

### Table 1. The sequences of primers used in quantitative the real-time polymerase chain reaction (qRT-PCR).

| Primer name       | Sequence (5’-3’)                  | Product size (bp) |
|-------------------|-----------------------------------|-------------------|
| MYH10-Forward     | CGACGCGTTGCAACACGCATC             | 371               |
| MYH10-Reverse     | GACACAGTTGATCTTCCAGGAAGG          |                   |
| MTA-1-Forward     | AGTACAGGACAGCACAACGGG            | 298               |
| MTA-1-Reverse     | CAGCCCTGGTTTCCCGAGG             |                   |
| MMP-2-Forward     | CAGCCCTGACAGTCCATT              | 210               |
| MMP-2-Reverse     | GTTGGCCAGGGAAATGAAGG            |                   |
| MMP-9-Forward     | GAGAGCTACCCACCCAGG             | 238               |
| MMP-9-Reverse     | GAAAGTGAAGGGGAAAGGGC            |                   |
| TIMP-1-Forward    | AGACACCTTATACACCGGT           | 217               |
| TIMP-1-Reverse    | GCCAAACACTGCGAGT            |                   |
| E-cadherin-Forward| TCCCTACAGCTGCCAGGA            | 321               |
| E-cadherin-Reverse| ATGGCTCTTGCTCCTCGT          |                   |
| Collagen I-Forward| CTTGTGACACAGGCCCCGC       | 247               |
| Collagen I-Reverse| GCTAAAAGACAGGCACTCCAG       |                   |
| Vimentin-Forward  | CAGCCATGACCACCCAG           | 1401              |
| Vimentin-Reverse  | AAGTGCAAAGGTCGCCAG           |                   |
| Wnt3a-Forward     | CAGATCCGGTTCCTCTTCTG          | 308               |
| Wnt3a-Reverse     | GTGATGAAATAGGCGACC             |                   |
| β-catenin-Forward | GGGTCTCTCTGGAACCTGT          | 316               |
| β-catenin-Reverse | AATCTTGGTGCTTCCTCA         |                   |
| Cyclin D1-Forward | CTGGCAGATCACTCCGGA         | 483               |
| Cyclin D1-Reverse | GTCACACTGATTACACCGG-3         |                   |
| GAPDH-Forward     | CCATCTCCAGGGAGCGAGAT        | 222               |
| GAPDH-Reverse     | TGCCTGATGACCTTGGAGGT        |                   |

MYH10 – myosin heavy chain 10; MMP – matrix metalloproteinase; MTA-1 – metastasis-associated protein 1; TIMP – tissue inhibitor of metalloproteinases.

as previously reported [18].
Western blot

Cells were lysed with liquid nitrogen and blocked with RIPA buffer (AbMole Bioscience, Houston, TX, USA), followed by 1% cleavage in phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitors (AbMole Bioscience, Houston, TX, USA) and lysis for 30 min at 4°C. The supernatant was collected by centrifugation at 12,000 rpm for 4 minutes at 4°C. A standard curve was drawn using the biocinonic acid (BCA) method to determine the protein concentration. The kits were purchased from Kaiji Biotechnology Development Co., Ltd. (Nanjing, China). A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared and the electrophoresis conditions were 80 V for 30 minutes. After completion of electrophoresis, the polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) was transferred using a Trans-Blot Transfer Slot (Bio-Rad, Hercules, CA, USA) and the membrane was transferred at 350 mV at 0°C for about 100 minutes. The membrane was removed and labeled.

The following primary antibodies were obtained from Abcam (Cambridge, MA, USA): anti-MYH10 (Abcam, ab6759) (1: 1000); anti-MTA-1 (Abcam, ab226960) (1: 1000); anti-MMP-2 (Abcam, ab168864) (1: 1000); anti-MMP-9 (Abcam, ab38898) (1: 1000); anti-E-cadherin (Abcam, ab6528) (1: 800); anti-vimentin (Abcam, ab92547) (1: 800); anti-collagen I (Abcam, ab34710) (1: 800); anti-Wnt3a (Abcam, ab28472) (1: 700); anti-β-catenin, (Abcam, ab32572) (1: 800); anti-cyclin D1 (Abcam, ab134175) (1: 800). The following primary antibodies were obtained from R&D Systems (Minneapolis, MN, USA): anti-TIMP-2 (MAB971) (1: 500); anti-GAPDH (MAB5718) (1: 800). The primary antibodies were added to the membranes according to the kit instructions, shaking at room temperature for 2 h, then incubated at 4°C for 12 h.

The secondary antibodies were added and incubated at room temperature for 1.5 h: rabbit anti-mouse IgG (Abcam, ab99697) (1: 10000); goat anti-mouse IgG (Abcam, ab6785) (1: 8000); mouse anti-rabbit IgG (BA1034) (1: 7000) (Invitrogen, Carlsbad, CA, USA); donkey anti-rabbit IgG (NL004) (1: 5000) (R&D Systems, Minneapolis, MN, USA); mouse anti-rabbit IgG (BA1034) (1: 7000) (Invitrogen, Carlsbad, CA, USA). Chemiluminescence detection was carried out using the enhanced chemiluminescence (ECL) reagent (Huiying, Shanghai, China).

Statistical analysis

All the experimental data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 20 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) following Tukey’s test was carried out to evaluate the differences between the experimental groups. Statistical significance was assumed to be a P-value <0.05.
Effects of siMYH10 on epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM)-related gene expression in T98G human glioma cells

To investigate the basis for the cell invasion and migration, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were used to detect the expression of extracellular matrix (ECM) and epithelial-mesenchymal transition (EMT)-related mRNA and proteins. There was no significant difference between the NC group and the control group. For the four EMT-related genes, the expression levels of metastasis-associated protein 1 (MTA-1), matrix metalloproteinase (MMP)2, and MMP9 genes and proteins were significantly reduced, while the expression of tissue inhibitor of metalloproteinases 2 (TIMP2) genes and proteins was increased (P<0.05). For the three ECM-related genes, the expression levels of E-cadherin and collagen I mRNAs and proteins were upregulated, while the expression level of vimentin was downregulated (P<0.01) (Figure 3A–3C).

Effects of siMYH10 on the expression of Wnt/β-catenin pathway genes in T98G human glioma cells

To investigate the mechanism of regulation of ECM and EMT after MYH10 silencing, the expression level of Wnt/β-catenin pathway related genes were studied. The results showed that the expression levels of Wnt3a, β-catenin, and cyclin D1 in the siMYH10 group were significantly lower compared with those of the NC group and the control group (P<0.01) (Figure 4A, 4B).

Discussion

The myosin heavy chain 10 or MYH10 gene encodes non-muscle myosin IIB (NM IIB) and is involved in tumor cell migration, invasion, the production of extracellular matrix (ECM) and in epithelial-mesenchymal transition (EMT). This study aimed to investigate the effects of the MYH10 gene on normal human glial cells and glioma cell lines in vitro, by gene silencing, and to determine the signaling pathways involved. Previous studies have shown that NM IIA and NM IIB can regulate cell movement by controlling cell polarization [19]. The findings of this study showed that the MYH10 gene was overexpressed in three glioma cell lines, U251, T98G, and SHG44, but that the expression level of MYH10 in the T98G cell line was the highest, which is why this cell line was identified for further study.

Previously published studies have shown that the overexpression of NM IIA protein was normal in many tumors [9,20]. NM IIA and NM IIB have a high degree of similarity, and studies have shown NM IIB is associated with malignant tumors [21], and that NM IIB is overexpressed in tumor cells [6]. By constructing an MYH10 interference plasmid and conducting transfection experiments, the expression level of the MYH10 gene was significantly reduced at 24 hours after transfection, but there was no significant effect on cell viability. This finding supported the success of the plasmid construction and transfection experiments and may provide a protocol for future studies.
MYH10-encoded NM IIB protein is closely related to cell division, cell adhesion, cell migration, and metastasis. The results of the 2006 study by Thiery and Sleeman showed that the MYH10 mutation caused changes in EMT that resulted in decreased cell migration [22]. A previous study has shown that EMT is involved in cell adhesions, contractility, and signaling [23]. The results of this study showed that after the inhibition of MYH10 expression, the migration and invasion of T98G human glioma cells were significantly reduced. Previous studies also found that NM IIB may play a role in cell adhesion during embryogenesis [24,25]. The presence of NM IIB has been shown to be involved in cell adhesion by maintaining the integrity of the epithelial zonula adherens [26]. In the study by Mai and colleagues, NMIIB was shown to play a role in tumor migration [27]. Therefore, in glioma, the findings that overexpression of the MYH10 gene was associated with invasion and migration of glioma cells, and inhibition of MYH10 gene expression could control the invasion and migration of glioma cells, are of great significance for the clinical treatment of glioma.

Figure 2. Effects of siMYH10 plasmid interference on the expression levels of MYH10 cell viability, cell motility, and cell invasion of the T98G human glioma cell line. T98G human glioma cells were transfected with phosphate-buffered saline (PBS) (the control group), without the siRNA plasmid (the NC group), and MYH10-siRNA plasmid (the siMYH10 group). (A) Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect the mRNA level of MYH10 in the different groups. (B) Western blot was used to assess the protein level of MYH10 in the different groups. (C) Cell viability was analyzed using the cell counting kit-8 (CCK-8) assay. (D) The cell scratch test was used to detect cell migration 24 hours after transfection the siMYH10 group). Scale bar, 50 mm. (E) The transwell assay was used to detect the rate of cell invasion 24 hours after transfection. Scale bar, 50 mm. * P<0.05, ** P<0.01, compared with the control group; * P<0.05, ** P<0.01, compared with the NC group. Data are shown as the mean ±SD (n=4).
Figure 3. Effects of transfection of siMYH10 plasmid on the expression of epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM)-related genes in the T98G human glioma cell line. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect mRNA of metastasis-associated protein 1 (MTA-1), matrix metalloproteinase (MMP)-1, MMP-9, tissue inhibitor of metalloproteinases 2 (TIMP2), E-cadherin, vimentin, and collagen I. (B) Western blot was used to detect the protein expression levels of MTA-1, MMP-2, MMP-9, and TIMP-2 proteins. (C) Western blot was used to detect the expression levels of E-cadherin, vimentin, and collagen I proteins. P<0.05, ** P<0.01, compared with the control group; ^ P<0.05, ^^ P<0.01, compared with the NC group. Data are shown as the mean ±SD (n=4).
cells is supported by findings from previous studies on other tissue and tumor types and requires further study in malignant glioma.

The migration and invasion of malignant cells are related to ECM and EMT processes [28]. E-cadherin, vimentin, and collagen I are common ECM proteins [29,30]. Metastasis-associated protein 1 (MTA-1), matrix metalloproteinase (MMP)2, and MMP9 are important components of the matrix metalloproteinase family, which can hydrolyze intercellular matrix proteins, providing cells with the ability to metastasize [31]. Tissue inhibitor of metalloproteinases 2 (TIMP2) can inhibit MMPs, while increased expression of E-cadherin, vimentin, and collagen I protein stabilize cells [32]. The migration and invasive ability of malignant cells have previously been shown to be associated with overexpression of MTA-1, MMP2, and MMP9 and inhibition of TIMP-2 expression [30–32]. The results of this study showed that after silencing of the MYH10 gene, the expression levels of MTA-1, β-catenin, and Cyclin D1 genes were significantly down-regulated, whereas TIMP-2 was up-regulated. These findings support that in gliomas, silencing the expression of MYH10 has the ability to reduce migration and invasion by increasing the expression of ECM-associated proteins and inhibiting EMT.

Clinical studies have confirmed that Wnt/β-catenin plays an important role in the development, migration, and invasion of cancer cells [33,34]. β-catenin is a key factor in this pathway. The Cyclin D1 gene is a proto-oncogene that maintains cells in the S-phase of cell proliferation resulting in increased cell proliferation [35]. The results of the present study showed that the relative expression levels of Wnt3a, β-catenin, and Cyclin D1 genes in T98G human glioma cells were reduced after MYH10 gene expression was inhibited.

Previous studies have shown that the Wnt pathway regulates the tumor microenvironment and promotes the transformation of epithelial cells and mesenchymal cells into cancer cells, thereby promoting tumor metastasis [36]. The dysregulation of Wnt/β-catenin signaling pathway can increase the migration and invasion of glioma cells [37]. The invasive ability of glioma cells has previously been shown to be reduced by inhibiting Wnt signaling through CBX7 overexpression, and it is possible that dysregulation of Wnt/β-catenin signaling can affect the expression of MMP2, MMP9, E-cadherin, N-cadherin and vimentin, and inhibit EMT and glioma invasion [38]. The Wnt protein includes many subtypes that activate the Wnt/β-catenin pathway, and Wnt3 is just one of these [38]. The Wnt3 protein has the effect of inducing differentiation of mesenchymal...
stem cells into neurons, and can also activate proteins in downstream pathways [39, 40]. The extracellular Wnt proteins bind to cell membrane receptors to inhibit the expression of glycogen synthase kinase 3β (GSK-3β), which results in the accumulation of β-catenin in the cell [41]. β-catenin as a critical protein in the Wnt/β-catenin pathway and has a role in tumor migration and invasion, as it can enter the nucleus and promote the expression of Cyclin D1 [42–44]. β-catenin/E-cadherin complexes are formed that promote cell adhesion, and after β-catenin enters the nucleus, it activates the T-cell factor/lymphoid enhancer factor-1 (TCF/LEF-1), which encodes vimentin and MMPs to promote EMT [42–44]. Wnt/β-catenin is also able to promote EMT through the mitogen-activated protein kinase (MAPK) signaling pathway [45]. As a cyclin protein, cyclin D1 overexpression increases cell proliferation, but few studies have been conducted on the role of cyclin D1 expression and cancer metastasis [35, 46, 47]. Although this was a preliminary in vitro study in human glioma cell lines, the findings showed that silencing the MYH10 gene reduced cell migration and invasion, by inhibiting the Wnt/β-catenin pathway, which may regulate the ECM and inhibit EMT in human glioma.

**Conclusions**

This study has shown that the MYH10 gene is overexpressed in human glioma cells in vitro, and that silencing the MYH10 gene reduced cell migration and invasion. These findings might be due to the reduction of extracellular matrix (ECM)-related protein levels and inhibition of epithelial-mesenchymal transition (EMT) by suppressing the Wnt/β-catenin pathway. Further in vivo studies should be undertaken to determine whether MYH10 may become a new target for the treatment of malignant glioma.

**Conflict of interest**

None.

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