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MYT1L promotes the migration and invasion of glioma cells through activation of Notch signaling pathway

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ABSTRACT: *MYT1L* gene has been associated with various brain diseases as it affects many neuronal and biological processes of nerve cells. However, the role of MYT1L in glioma cell migration and invasion and its mechanism of action are still unclear. In this study, MYT1L was successfully silenced in U87 and A172 cells, and overexpressed in U251 cells using lentiviral vectors. The expression levels of MYT1L in glioma cells were assessed by real-time PCR and Western blot analysis. The effect of MYT1L on migration and invasion was examined by a Transwell assay. We found that MYT1L enhanced cell migration and invasion whereas knockdown of MYT1L suppressed migration and invasion in glioma cells. We also demonstrated that MYT1L activated the Notch signaling pathway, and that treatment with the Notch inhibitor DAPT inhibited the migration and invasion of glioma cells. These results suggest that MYT1L may be considered a useful and potential target in the treatment of glioma.

KEYWORDS: glioma, MYT1L, migration, invasion, Notch signaling

INTRODUCTION

Gliomas are the most common malignant brain tumors in adults [1] especially in developing countries [2]. The current treatment for gliomas is surgery combined with chemotherapy and/or radiotherapy [1, 3]. Although diagnostic and therapeutic strategies for gliomas have improved greatly in recent decades, some types of gliomas (such as glioblastoma) having a relative survival of 5 years are not exceeded 5% due to the serious resistance to these interventions and high frequency of recurrences [1]. Therefore, it is essential to thoroughly and comprehensively investigate the original signaling pathways and novel molecular mechanisms of tumorigenesis in gliomas.

Myelin transcription factor 1 (*MYT1*) encodes a member of the zinc finger superfamily of transcription factors expressed primarily in the developing central nervous system (CNS). *MYT1* plays a critical role in neuronal differentiation by specifically inhibiting the expression of non-neuronal genes during the differentiation of neurons and is involved in the initiation and development of many nervous system diseases [4, 5]. For example, *MYT1L* variants are associated with human intellectual disability and syndromic obesity [6]. *MYT1L* was considered a potential candidate gene for fibromyalgia in the study by D'Agnelli [7]. The groups of Wang [8] and Lee [9] confirmed that *MYT1L* is associated with autism and schizophrenia. In addition, a study by Hu et al [4] showed that tumorigenesis can be profoundly inhibited when *MYT1L* is reintroduced into glioma stem cells, and that knockdown of *MYT1L* in premalignant neural stem cells inversely promotes orthotopic tumor formation. Moreover, the proliferation of GBM cells can be limited by suppression of YAP1 expression caused by affecting MYT1L and MYT1L transcription factors [10]. Surprisingly, downregulation of MYT1L resulted in decreased proliferation in Neuro2a blastoma cells, an effect that was also observed upon downregulation of the MYT1L interacting lysine demethylase LSD1 [11]. However, the effects of MYT1L on Notch signaling and the mechanism of MYT1L involvement in glioma cell migration and invasion remain unclear.

MATERIALS AND METHODS

Cell culture and transfection

Human glioma cell lines (U87, A172, and U251) and HEK293T cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U87 cells were grown in MEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA). A172, U251, and HEK293T cells were cultured in DMEM medium (Thermo Fisher Scientific) containing 10% FBS. All cells were incubated in a humidified atmosphere at 37 °C and 5% CO_2. Lentiviral vector expressing *MYT1L* gene (pTight-hMYT1L-N174) was obtained from Addgene (plasmid#31877). Lentiviral vectors expressing short hairpin RNAs (shRNAs) targeting *MYT1L* were purchased from Shanghai Genechem Co., Ltd. They were shMYT1L#1 (target sequence: 5′-TCG TTTGAATACAACAGTT-3′), shMYT1L#2 (5′-ATCGCTT

TGGAAACGGAAA-3′), shMYT1L#3 (5′-AGCAAGACA GTAGAAATAT-3′), and shMYT1L#4 (5′-AAAGCCATT TGCCGTGAAA-3′). Control shRNA was shLuc (target *Luciferase* gene) constructed earlier in our laboratory. Lentiviral particles were generated by co-transfecting pMDLg/pRRE, pRSV-Rev, and pMD2.G envelope plasmid and the lentiviral expression vectors using polyethylenimine (Sigma-Aldrich). U87 and A172 cells were transduced with lenti-shMYT1L to stably knockdown the expression of MYT1L. U251 cells were transduced with lenti-MYT1L to establish a model of MYT1L overexpression, and cells with lenti-GFP were considered as a control group.

Real-time PCR

Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific). Complementary DNA synthesis was synthesized using the PrimeScript RT Reagent Kit (Takara, Japan), and mRNA expression levels were detected with the TB Green Premix Ex Taq (Takara) on the QuantStudio 5 real-time PCR system (Applied Biosystems, Warrington, U.K.). The real-time primers used in this study were: *MYT1L*-F: 5′-GAGCAGATG CTGACCATCAA-3′, *MYT1L*-R: 5′-TCGTGGAGGAGAGA CTCGTT-3′. GAPDH was considered as an internal control.

Western blot analysis

Cells were lysed in RIPA buffer containing protease and phosphate inhibitors. Proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were blocked with 5% fat-free milk in TBST (Tris-buffered saline, 0.1% Tween 20). Then, the membranes were incubated with dilutions of primary antibodies overnight at 4 °C. The results were detected with enhanced chemiluminescence in the Imaging Analysis System (Bio-Rad, Hercules, CA, USA). The following primary antibodies were used: Anti-MYT1L was purchased from Abcam; anti-NOTCH1, anti-HES1, anti-JAG1, anti-HES5, and anti-β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated secondary antibodies were from Multi Sciences (Hangzhou, China).

Cell proliferation

For the proliferation assay, cells were seeded in 96-well plates at 100 µl/per well and incubated at 37 °C for five days. Cell viability was determined using the cell counting kit-8 (CCK-8) assay (Dojindo, Japan). Briefly, 10 µl CCK-8 solutions were added to each well at the indicated time point and incubated for 3 h. The absorbance value was measured at 450 nm using a spectrophotometer.

Migration and invasion assays

The cell migratory and invasive abilities were determined by a transwell chamber assay using 24-well Transwell units (Corning, NY, USA) and a polycarbonate filter with a pore size of 8 µm. For migration assays, cells (1×10^5) were seeded in the upper chamber in FBS-free MEM medium. MEM medium containing 20% FBS was added to the lower chamber. After incubation for 24 h at 37 °C, cells in the chambers were fixed with 4% paraformaldehyde and then determined by crystal violet staining assay. The remaining cells on the top of the chamber were removed with a cotton swab. The number of cells reaching the lower chamber was counted using an inverted microscope. For invasion assays, the membrane was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to form a continuous thin layer. To investigate the effects of DAPT treatment on cell migration and invasion, cells were treated with DAPT or a control for 48 h in a Transwell insert.

Statistical analysis

All experiments were performed in triplicate and results were summarized as mean ± standard deviation (SD). Student's t-test or one-way analysis of variance was used to calculate differences between two or three groups. $p < 0.05$ was considered statistically significant.

RESULTS

Knockdown of MYT1L by shRNA inhibits migration and invasion of glioma cells

The protein levels of MYT1L in three glioma cell lines (U87, A172, and U251) were detected by Western blotting. U87 and A172 cells showed relatively high expression of MYT1L, whereas U251 cells showed relatively low expression of MYT1L (Fig. 1a). Four shRNAs targeting different sites were constructed and the shMYT1L#2 with the best knockdown was then selected for further study (Fig. 1b). Fig. 1c and 1d show that the mRNA and protein levels of MYT1L were significantly suppressed in U87 and A172 cells transduced with lenti-shMYT1L. Transwell assays showed that knockdown of MYT1L expression significantly inhibited cell migration and invasion in U87 (Fig. 1e) and A172 (Fig. 1f) cells, and the experiment was repeated three times.

MYT1L activates the Notch signaling pathway in glioma cells

MYT1L was overexpressed in U251 cells, which expressed a relatively low level of MYT1L. Overexpression of MYT1L in U251 was detected by real-time PCR and Western blot (Fig. 2a and 2b). Compared with the control group, the migration and invasion abilities of the overexpression group were significantly increased in Tranwell assays (Fig. 2c). Fig. 3 shows that the protein levels of NOTCH1, JAG1, HES1, and HES5 were significantly suppressed in U87 and A172 cells transduced with lenti-shMYT1L, whereas the levels of

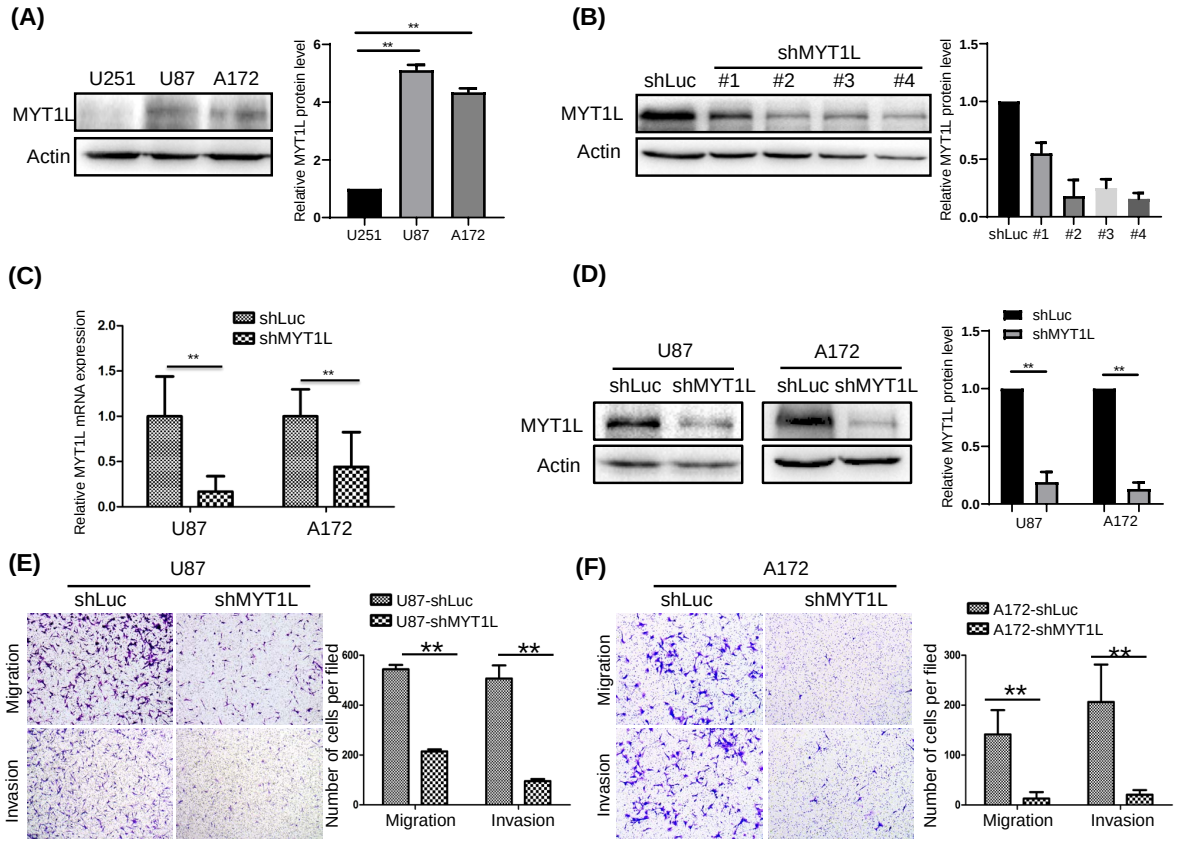

Fig. 1 Effects of MYT1L knockdown on glioma cell migration and invasion. (a) Protein expression level of MYT1L in glioma cell lines U87, A172, and U251 determined by Western blot. (b) Western blot analysis of MYT1L expression in U87 cells transduced with lenti-shMYT1L. The shMYT1L#2 with the best knockdown was selected for further experiments. (c) mRNA level of MYT1L in U87 and A172 cells transduced with control (lenti-shLuc) or lenti-shMYT1L determined by real-time PCR. (d) Protein level of MYT1L in U87 and A172 cells transduced with control (lenti-shLuc) or lenti-shMYT1L determined by Western blot. (e-f) Migratory and invasive abilities of U87 and A172 cells transduced with control (lenti-shLuc) or lenti-shMYT1L assessed by Transwell assays. $^{**}p < 0.01$.

these proteins were significantly increased in U251 cells with MYT1L overexpression.

Inhibition of Notch signaling by DAPT suppresses cell migration and invasion in glioma cells

DAPT, a γ-secretase inhibitor, can inhibit Notch signaling and block transcription of downstream genes, resulting in cellular dysfunction [12]. To investigate the effect of Notch signaling on cell growth, migration, and invasion, U87 and A172 cells were treated with DAPT. CCK-8 assays showed that inhibition of Notch signaling by DAPT had no effect on cell growth (Fig. 4a and 4b). However, DAPT treatment significantly inhibited the migration and invasion ability of U87 and A172 cells (Fig. 4c and 4d), and the experiment was repeated three times.

DISCUSSION

Gliomas remain the most common primary malignant tumors of the nervous system. Approximately 42% of all tumors occur in men and 58% in women [3]. Although the treatment of gliomas has greatly improved, their mortality rate is still high worldwide due to inadequate studies of the tumors. Limited number of researches on the molecular mechanism has resulted in low efficiency in the treatment of patients with gliomas [13]. In the current study, we found that MYT1L was expressed in glioma cell lines U251, U87, and A172. The expression of MYT1L was upregulated in U251 cells and downregulated in U87 and A172 the cells by lentiviral vectors. The migration and invasion in glioma cells were promoted by the overexpression

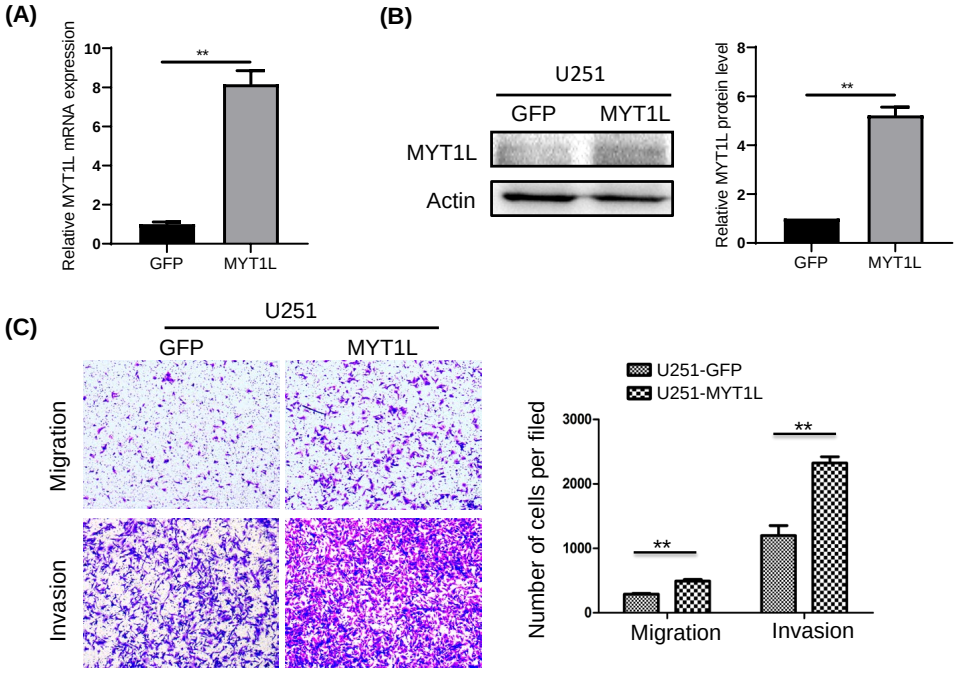

Fig. 2 Effects of MYT1L overexpression on glioma cell migration and invasion. The U251 cells were transduced with lenti-GFP (control) or lenti-MYT1L. (a) mRNA level as determined by real-time PCR. (b) Protein level determined by Western blot. (c) Migratory and invasive abilities assessed by Transwell assays. ** $p < 0.01$.

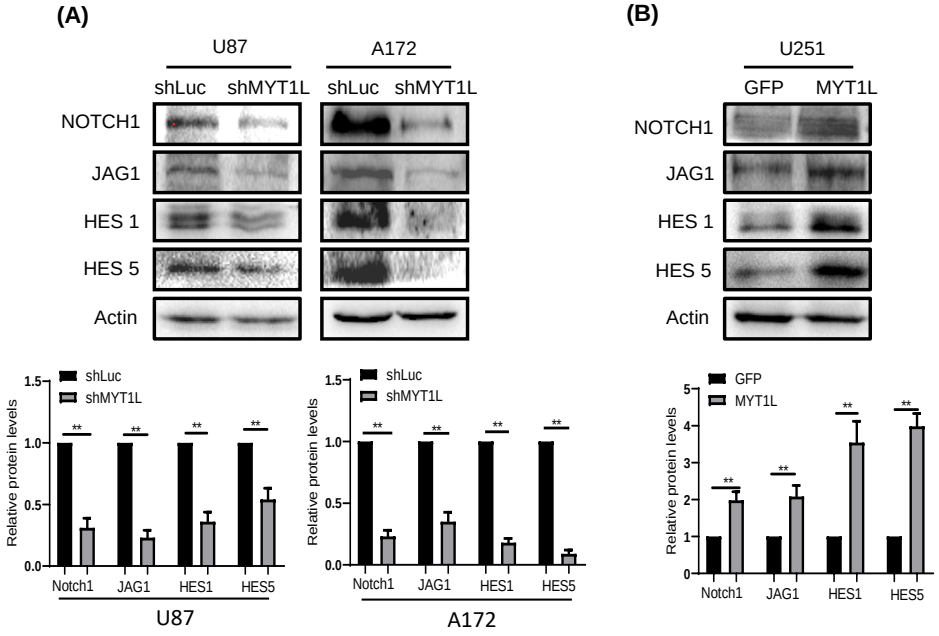

Fig. 3 Effects of MYT1L overexpression and knockdown on Notch1 signaling pathways, as determined by Western blot. (a) Protein expression levels of NOTCH1, Jagged1 (JAG1), HES1, and HES5 in U87 and A172 cells transduced with control (lenti-shLuc) or lenti-shMYT1L. (b) Protein expression levels of NOTCH1, JAG1, HES1, and HES5 in U251 cells transduced with lenti-GFP (control) or lenti-MYT1L. ** $p < 0.01$.

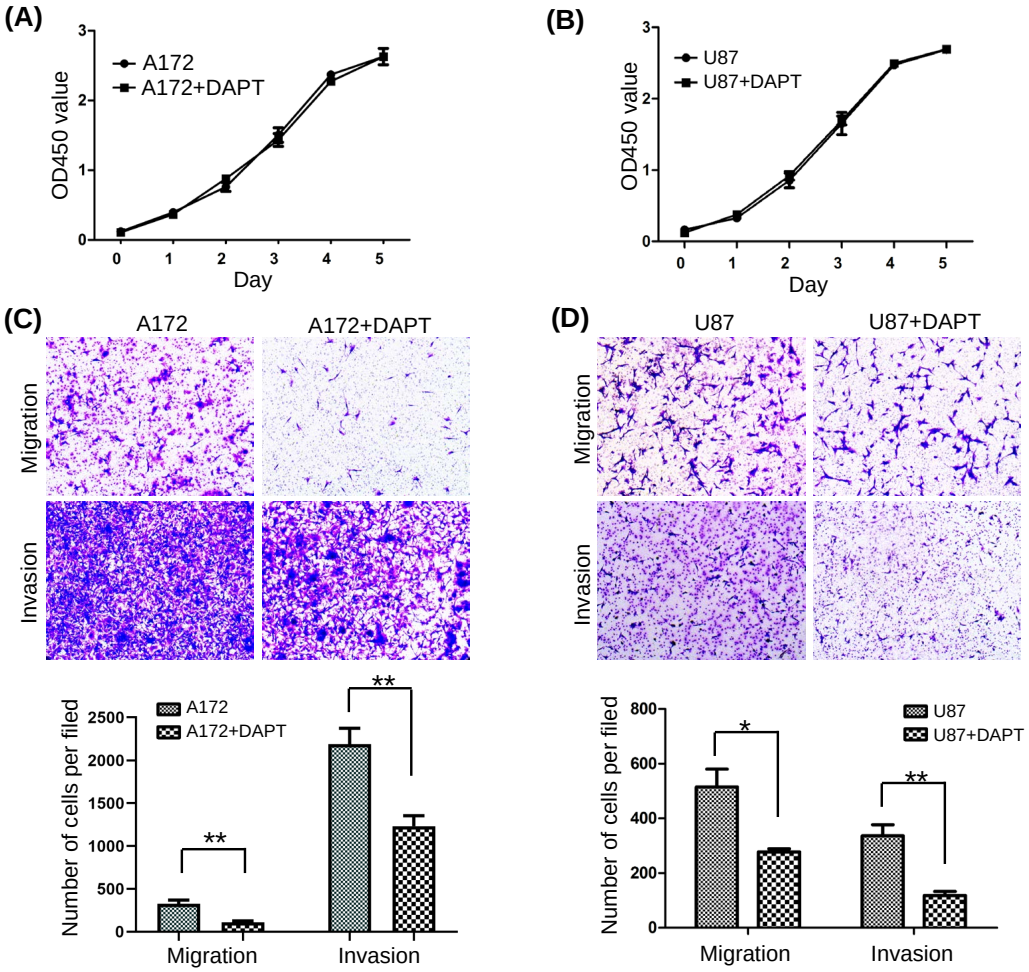

Fig. 4 Effects of γ-secretase inhibitor (DAPT) on glioma cell migration and invasion. U87 and A172 cells were treated with control or DAPT. (a-b) Cell proliferation assessed by CCK-8 assays. (c-d) Migratory and invasive abilities assessed by Transwell assays. $^{*}p < 0.05$; $^{**}p < 0.01$.

of MYT1L but inhibited by the knockdown of MYT1L.

Previous studies have shown that MYT1L, which is related to transcription factors, plays a role in both transcriptional activation and repression of gene expression [14], with implications for tumors. For example, a study by Zhang et al [15] suggested that MYT1L is associated with the prognosis of gastric cancer. Cheng et al [16] found that MYT1L can affect the regrowth of a nonfunctioning pituitary adenoma. In addition, MYT1L may indicate poor prognosis in medulloblastoma [17].

The Notch signaling pathway has been associated with stem cell fate determination and cancer [18]. Inhibition of the Notch signaling pathway by γ secretase inhibitors, such as DAPT, reduces tumor neurosphere growth [19], glioblastoma xenograft initiation, and clonogenic growth *in vivo* [20]. In mammals, the Notch receptor family includes the Notch 1–4 receptors, whose ligands include Delta-like-1(DLL1), Delta-like-3(DLL3), Delta-like-4(DLL4), Jagged-1(JAG1), and Jagged-2(JAG2). Activation of the Notch pathway requires receptor binding to ligands and proteolytic hydrolysis to generate the intracellular Notch domain, which is released into the nucleus and activates downstream HES and HEY via a series of reactions, leading to corresponding biological effects [21]. Many studies show that the Notch signaling pathway plays a critical role in tumorigenesis in many tumors [21–24]. It has been demonstrated that upregulation of JAG1 and activation of the Notch signaling pathway can promote metastasis of U251 glioma cells via Yes-associated protein 1 [25]. Taylor et al [26] detected the level of Notch effectors HES1, HES4, and HES5 in diffuse intrinsic pontine glioma cells treated with the γ-secretase inhibitor MRK003 and found that Notch may be an effective therapeutic strategy for diffuse

intrinsic pontine gliomas. Notch signaling activity was increased in glioblastoma multiforme tissues and promotes invasion, self-renewal and growth of glioma initiating cells [27]. Therefore, in our study, the proteins of NOTCH1, JAG1, HES1, and HES5 in glioma cells with MYT1L overexpression or knockdown were detected by Western blot. We found that MYT1L could activate the Notch1 signaling pathway. However, the roles of other Notch receptors and ligands remain to be further investigated. In addition, we found that inhibition of the Notch signaling pathway by the γ secretase inhibitor DAPT suppressed cell migration and invasion in glioma cells. These results suggested that the mechanism of MYT1L promoted cell migration and invasion in gliomas and may lie in the activation of Notch signaling.

In conclusion, our results demonstrate that MYT1L activates the Notch signaling pathway and promotes cell migration and invasion in gliomas. Treatment with the Notch inhibitor DAPT inhibited glioma cell migration and invasion. These data suggest that MYT1L might be a useful and potential target in the treatment of gliomas. The discovery and the characterization of MYT1L function and the NOTCH signaling pathways represent an opportunity to enhance our understanding of the molecular mechanisms involved in glioma progression.

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