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*Original Article*

# Claudin 4 enhances the malignancy of glioma cells via NNAT/Wnt signaling

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**Abstract:** Gliomas are the most common malignancies of the central nervous system and are associated with high mortality rates. However, the pathogenesis of gliomas is unclear. In this study, we show that elevated claudin-4 (CLDN4) levels in glioma tissues are associated with poor clinical outcomes. We found that upregulating the expression of CLND4 enhanced the proliferative and migratory capacities of glioma cells. Mechanistically, CLND4 upregulated Neuronatin (NNAT) by activating Wnt3A signaling, and aided in the progression of the glioma. Most importantly, our *in vivo* data demonstrated that CLND4 overexpression caused rapid tumor growth in mice injected with LN229 cells and reduced the survival of these mice. Our findings reveal that CLND4 modulates malignancy in glioma cells; targeting CLDN4 may open up new avenues for glioma treatment.

**Keywords:** Claudin 4 (CLDN4), glioma, neuronatin (NNAT), Wnt signaling

## Introduction

Gliomas are highly aggressive brain tumors characterized by a high degree of heterogeneity and mortality. Currently, the standard of care for glioma includes maximal safe resection, radiotherapy, and treatment with temozolomide (TMZ) [1]. Despite advances in therapeutic modalities, patients with gliomas continue to have dismal prognoses [2]. The limited efficacy of most therapies is in part due to the aggressive characteristics of glioma cells that support metastasis and tumor relapse [3]. Therefore, it is critical to understand the molecular pathways involved in glioma occurrence and progression.

Tumor metastasis requires a complex succession of events that include invasion, intravasation, and extravasation [3]. The process of invasion occurs when neoplastic cells lose cell-to-cell adhesion and disengage from the primary tumor mass. Tight junctions (TJs), which create a seal between adjacent polarized epithelial or endothelial cells, are therefore significant barriers against tumor invasion [4]. A growing body of literature has linked failures in the maintenance of TJs to malignant transformation [5, 6]. The main constituent membrane proteins of TJs are claudins. Claudins are a multigene family of proteins with 27 members; these genes maintain cell polarity and establish a selective permeability barrier between cells and control the fluxes of molecules between the cells [7]. Claudins may also modulate cellular behaviors by interacting with signaling proteins [8]. The aberrant expression of claudins in many malignancies is known to have pro- or anti-tumorigenic effects [9, 10]. For example, in renal cell carcinoma, the progressive loss of claudin-2 (CLDN2) expression was correlated with poor patient survival, whereas CLDN2 overexpression retarded tumor growth in xenograft mice models [11]. Katsushima et al. have proposed that the upregulation of claudin-11 (CLDN11) expression may be required for glioma cell differentiation and establishment of intra-tumoral heterogeneity [12]. Several lines of evidence point to claudin-4 (CLDN4) as being important for the overall survival of patients due to its

dual function in tumor progression [13]. In a study on pancreatic cancer, CLDN4 overexpression restricted pulmonary metastasis *in vivo*; the overexpression also promoted the formation of more cell-to-cell contacts [14]. In addition, CLDN4 overexpression enhanced the sensitivity of gastric cancer cells to chemotherapy by suppressing the PI3K/Akt pathway [15]. Despite these studies, the role of the claudin family of proteins in the development and progression of gliomas remains to be investigated in detail.

In this study, we found that CLDN4 levels were positively correlated with the clinical stages of glioma patients. Our *in vitro* data demonstrates that increased CLDN4 expression accelerated glioma cell proliferation and migration. In addition, CLDN4 overexpression was associated with an increase in the sizes of tumors in glioma xenograft models. We have also found that CLND4 affects the progression of gliomas through its effects on the NNAT/Wnt signaling pathway. The tumor-promoting role of the CLND4/NNAT axis was verified in glioma organoids. Taken together, our results suggest that CLDN4 may be a useful biomarker and potential therapeutic target for the treatment of gliomas.

## Materials and methods

### *Cell culture*

The human glioma cell lines, LN229 and T98G, were obtained from the American Type Culture Collection, and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher, USA) supplemented with 10% fetal calf serum (Thermo Fisher). The cells were cultured at 37°C, 5% $CO_2$. Transfected LN229 and T98G cells overexpressing CLDN4 were obtained from GenScript ProBio (China); the same cells transfected with the empty vector (without the CLDN4 gene) were also obtained from the same source. The levels of the CLDN4 protein in these cells were determined by western blotting before the experiments. The Wnt signaling pathway inhibitor, pyrvinium pamoate, was obtained from Yeasen (China).

### *Clinical specimens and patients-derived organoids (PDOs)*

Data on the expression levels of CLDN4 in glioma tissues (n=156) and adjacent normal tissue samples (n=5) were obtained from http://ualcan.path.uab.edu/index.html. We also obtained data on 513 glioma patients from the same source for survival and correlation analysis. A total of 15 para-carcinoma tissues and 30 glioma tissues (early stage or stage I-II (n=15) and late stage or stage III-IV (n=15)) were obtained from West China Hospital, Sichuan University. For culturing PDOs, fresh glioma tissues were minced into 1-2 $mm^3$ pieces and digested in DMEM (Thermo Fisher, USA) containing type IV collagenase (Sigma, UK) and DNase I for 2 h. Following this, a 40 μm cell strainer (Thermo Fisher, USA) was used for filtering out the disaggregated cell suspension. After lysing the red blood cells in the mixture, the glioma cells were seeded into 3D Matrigel containing a specific culture medium and required growth factors (1 mL DMEM: 1 mL F12 medium, 1 mL Neurobasal medium, 0.002 mL 100 × Glutamax, 0.002 mL 100 × low-glutamate non-essential amino acids mixture; Thermo Fisher, USA) [16]. All experiments were approved by the ethics committee of West China Hospital, Sichuan University and were conducted as per the Declaration of Helsinki.

### *Cell proliferation and colony formation assay*

Cell proliferation was measured with a Cell Counting Kit-8 (CCK-8; Solarbio, China). The single cell suspensions ($1 \times 10^3$ per well) were cultured in a 96-well plate for 0, 24, 48 and 72 h. Following this, 10 μL of the CCK-8 reagent was added to each well and incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader (Biorad, USA).

For colony formation assays, the glioma cells were seeded into 6-well plates at a density of 500 cells/well and incubated at 37°C overnight. After 10 days, the cell colonies were fixed with 100% methanol, stained with 0.1% violet, and counted using a light microscope (Nikon, Japan). Any cluster of cells with >50 cells was considered a colony.

### *Cell migration assay*

For Transwell assays, $1 \times 10^5$ glioma cells were seeded into the upper chamber (8 μm, Coring, USA) of the Transwell assay system. The lower chamber was filled with 1 ml of culture medium supplemented with 10% fetal calf serum. After 24 h of incubation at 37°C, the adherent cells

on the lower surface of the insert were stained with Giemsa (Solarbio, China) and counted using a light microscope (Nikon, Japan).

*RNA interference*

For this experiment, either LN229 or T98G cells were seeded into 6-well plates at a density of 2 × $10^5$ cells per well. The cells were incubated without serum for 4 h at 37°C. The cells were transfected with siRNAs targeting the human NNAT and Wnt3A genes (Ruibo, China) using lipofectamine 3000 (Thermo Fisher, USA) as per the manufacturer's instructions. Silence efficiency was determined by real-time quantitative polymerase chain reaction (NNAT, siRNA #1, 73.4% and siRNA #2, 69.5%).

*Western blotting*

The expression levels of the CLDN4, NNAT, Wnt1, Wnt2, and Wnt3A proteins were assessed by standard western blot analysis. Anti-CLDN4 antibody (ab210795, Abcam, UK), anti-NNAT antibody (ab27266, Abcam, UK), anti-Wnt1 antibody (ab15251, Abcam, UK), anti-Wnt2 antibody (ab109222, Abcam, UK) and anti-Wnt3A antibody (ab219412, Abcam, UK) were diluted in TBST supplemented with 2% bovine serum albumin and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Blots were visualized using enhanced chemiluminescence and exposed to hypersensitive chemiluminescence film.

*Immunostaining*

Sections of the tissue specimens or Matrigel layers (containing the PDOs) were treated with 2.5% $H_2O_2$ and blocking buffer (5% bovine serum albumin) for 30 min at room temperature. Subsequently, the samples were incubated with primary antibodies: Anti-CLDN4 antibody (ab210795, Abcam, UK), anti-NNAT antibody (ab27266, Abcam, UK) and anti-Wnt3A antibody (ab219412, Abcam, UK) overnight at 4°C. Following this, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The sections were then visualized and analyzed using either an optical microscope (Leica, Germany) or a confocal microscope (Olympus, Germany). The protein expression levels were quantified using the Image-Pro Plus 5.1 software (Media Cybernetics, USA).

*Animal protocols*

Female NOD-SCID mice (6-8 weeks) were purchased from Huafukang (Beijing, China) and raised in a specialized pathogen-free facility. The mice were subcutaneously injected with 5 × $10^5$ LN229 cells (which were either overexpressing CLDN4 or were transfected with the empty vector; n=5 in each group). Tumor volume and survival information were recorded daily. Tumor volume was calculated as length × width$^2$/2. All animal experiments were approved by the Institute Ethics Committee of West China Hospital, Sichuan University, and complied with the ARRIVE guidelines.

*Statistical analysis*

All experimental data were analyzed using GraphPad Prism 5.0. The data are presented as mean ± SD. Comparisons between any two groups were done using an independent sample t-test. Comparisons for multiple groups were done using one-way ANOVAs, followed by Tukey's post-hoc tests. The overall survival of patients was analyzed using the Kaplan-Meier statistic. All experiments were independently performed at least thrice. Results were considered statistically significant at $P<0.05$.

## Results

*CLND4 promoted the development of malignant characteristics in gliomas and is associated with poor prognosis*

To investigate the role of CLDN4 in the progression of gliomas, we evaluated the expression levels of CLDN4 in the transcriptomes of 156 glioma tissue samples and compared them with those of normal tissues; these data were obtained from TCGA database. We found that CLDN4 expression was higher in glioma tumors than in normal tissues (**Figure 1A**). Immunohistochemical staining of tissue specimens also demonstrated that the proteins levels of CLDN4 were higher in glioma tissues as compared to those in para-carcinomatous tissues. We also found that the expression levels of CLDN4 were positively correlated with the clinical stage of the glioma (**Figure 1B**). Subsequently, we also found that patients with gliomas having low expression levels of CLDN4 had longer survival times than patients with gliomas having high CLDN4 expression levels

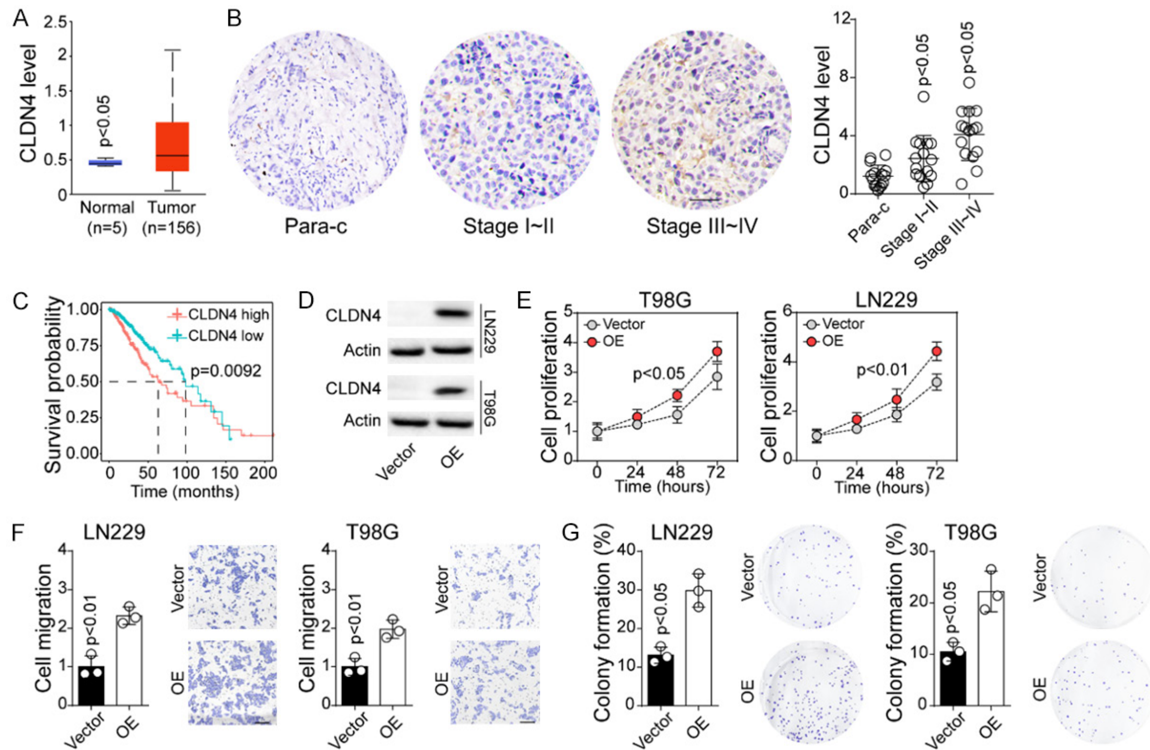


**Figure 1.** CLND4 promotes malignancy in glioma cells. A. Expression levels (mRNA) of CLDN4 in normal tissues (n=5) and glioma tissues (n=156); data derived from TCGA database. B. Representative images of immunohistochemical staining for CLDN4 in para-carcinomatous tissues (n=15), early-stage glioma tissues (stage I-II, n=15), and late-stage glioma tissues (stage III-IV, n=15); scale bar =50 μm. C. Kaplan-Meier survival curve for high and low expression of CLDN4 in glioma patients (n=513); data from TCGA database. D. Western blotting for CLDN4 in LN229/T98G cells transfected with empty vector or a CLDN4-overexpression vector. E. Cell proliferation assay (CCK-8 assay) for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector. T98G, P<0.05, 72 hours; LN229, P<0.01, 72 hours. F. Cell migration assay (Transwell assay) for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector; scale bar =100 μm. G. Colony formation assay for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector.

(**Figure 1C**), indicating that CLDN4 expression was positively correlated with glioma progression. To further investigate the pro-tumorigenic effects of CLDN4, we overexpressed CLDN4 in the glioma cell lines LNN229 and T98G (**Figure 1D**). The cells overexpressing CLDN4 had higher cell proliferation rates (**Figure 1E**), migration capacities (**Figure 1F**) and colony formation abilities (**Figure 1G**) than those not overexpressing CLDN4 (vector group). Together, these data indicate that CLDN4 promotes the development of malignant characteristics and is associated with poor prognosis in patients with gliomas.

*CLDN4 stimulates the expression of NNAT*

Next, we investigated the molecular pathway through which CLDN4 affects glioma progression. We used data on 513 glioma patients from TCGA database for this analysis; the patients were divided into CLDN4-high and CLDN4-low groups, and differences in their gene expression patterns were analyzed. The top 30 genes which were found to be upregulated in the CLDN4-high group as compared to the CLDN4-low group were identified (**Figure 2A**). Among these genes, we identified NNAT, which is a stress-response protein found in the rod photoreceptors. Since high NNAT expression is associated with poor outcomes in breast cancer [17], we performed western blotting assays to ascertain the expression levels of NNAT in glioma cells overexpressing CLDN4. We found that both, the RNA and protein levels of NNAT were higher in the LN229 and T98G cells that overexpressed CLDN4 as compared to those that did not overexpress CLDN4 (**Figures 2B** and S1A). In addition, we treated the LN229/T98G cells overexpressing CLDN4

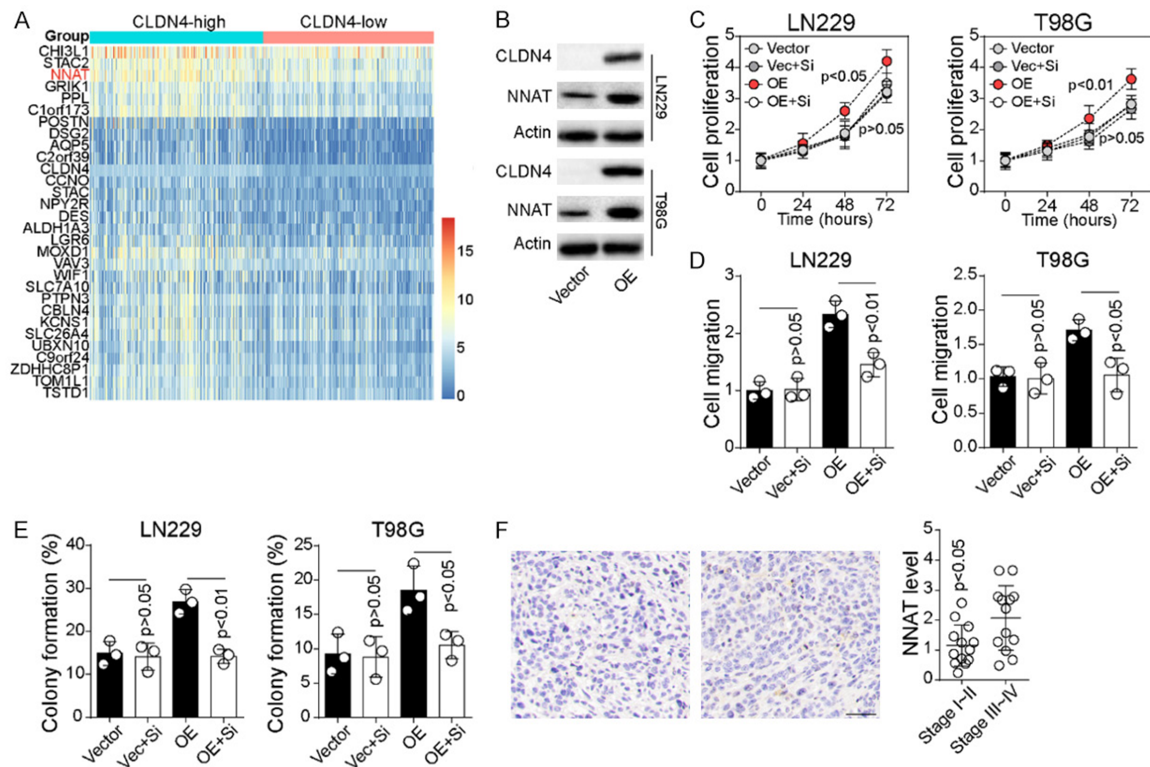


**Figure 2.** CLDN4-stimulated NNAT upregulation. A. The top 30 upregulated genes in patients with gliomas that had high CLDN4 expression levels as compared to those with gliomas having low CLDN4 expression levels; data derived from TCGA database (n=513). B. Western blotting for CLDN4 and NNAT in LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector. C. Cell proliferation assays for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with scramble or NNAT siRNAs. LN229, vector vs vector + siRNA, P>0.05, OE vs OE + siRNA, P<0.05, 72 hours; T98G, vector vs vector + siRNA, P>0.05, OE vs OE + siRNA, P<0.01, 72 hours. D. Cell migration assay for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with scramble or NNAT siRNAs. E. Colony formation assays for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with scramble or NNAT siRNAs. F. Representative images of immunohistochemical staining for CLND4 in early-stage glioma tissues (stage I-II, n=15) and late-stage glioma tissues (stage III-IV, n=15); scale bar =100 μm.

with siRNA targeted at NNAT. The knockdown of NNAT efficiently suppressed the cell proliferation (**Figure 2C**), migration (**Figure 2D**), and colony formation abilities (**Figure 2E**) of the glioma cells overexpressing CLDN4. We further found that the tumor tissues of advanced-stage gliomas (stages III-IV) had higher protein levels of NNAT as compared to those from earlier stages (stages I-II, **Figure 2F**). Collectively, those findings suggested that CLDN4 upregulates the expression of NNAT, which in turn, promotes glioma progression.

*The CLDN4/NNAT axis modulates glioma progression through Wnt signaling*

To further elucidate how the CLDN4/NNAT axis regulates the pro-survival signaling in gliomas, we analyzed the major signaling pathways associated with CLDN4 and NNAT. A KEGG enrichment analysis was performed to determine the major pathways involved in the CLDN4- (**Figure 3A**) and NNAT-mediated progression in gliomas (**Figure 3B**). Both, CLDN4 and NNAT, were closely associated with the Wnt signaling pathway. To validate the role of Wnt signaling in CLDN4-induced glioma progression, we examined the expression levels of Wnt1, Wnt2, and Wnt3A in the LN229 cells overexpressing CLDN4. We found that CLDN4 overexpression greatly upregulated the levels of Wnt3A in these cells (**Figure 3C**). We also found that silencing the NNAT gene (using anti-NNAT siRNA) suppressed the upregulation of Wnt3A expression in these cells (**Figure 3D**); this indicates that the CLDN4/NNAT axis stimu-

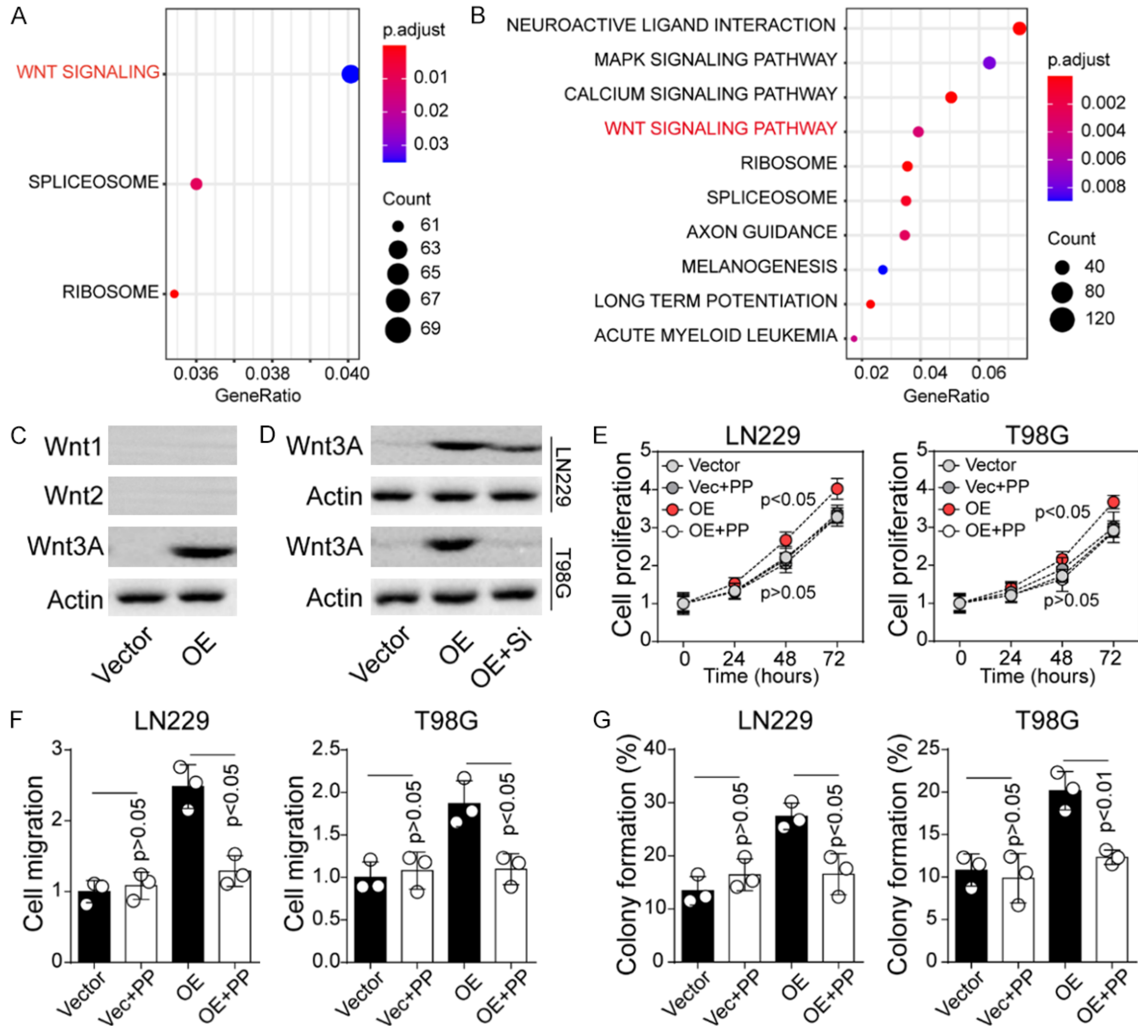


**Figure 3.** The CLDN4/NNAT axis modulates glioma progression through Wnt signaling. (A and B) The results of the KEGG enrichment analysis for the major signaling pathways involved in glioma progression in 513 glioma patients divided into CLDN4-high/low (A) or NNAT-high/low (B) groups. (C) Western blotting for Wnt1, Wnt2, and Wnt3A in LN229 cells transfected with empty vector or CLDN4-overexpression vector. (D) Western blotting for Wnt3A in LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with scramble or NNAT siRNAs. (E) Cell proliferation assays for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with PBS or pyrvinium pamoate (10 nM). LN229, vector vs vector + PP, P>0.05, OE vs OE + PP, P<0.05, 72 hours; T98G, vector vs vector + PP, P>0.05, OE vs OE + PP, P<0.05, 72 hours. (F) Cell migration assays for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with PBS or pyrvinium pamoate (10 nM). (G) Colony formation assays for LN229/T98G cells transfected with empty vector or CLDN4-overexpression vector treated with PBS or pyrvinium pamoate (10 nM).

lates Wnt3A signaling in glioma cells. Subsequently, we treated cells overexpressing CLDN4 with pyrvinium pamoate, which is an inhibitor of Wnt signaling. Blocking the Wnt signaling pathway efficiently suppressed cell proliferation (**Figure 3E**), migration (**Figure 3F**), and colony formation (**Figure 3G**) in the cells overexpressing CLDN4. Similar results were found in glioma cells treated with the Wnt inhibitor (Figure S1B-E). These results suggest that the CLDN4/NNAT-mediated progression in gliomas is dependent on the Wnt signaling pathway.

*CLDN4 facilitated glioma growth in vivo*

To investigate the effects of CLDN4 on glioma growth *in vivo*, we isolated primary human glioma cells and examined the expression of CLDN4 in these cells. As shown in **Figure 4A**,

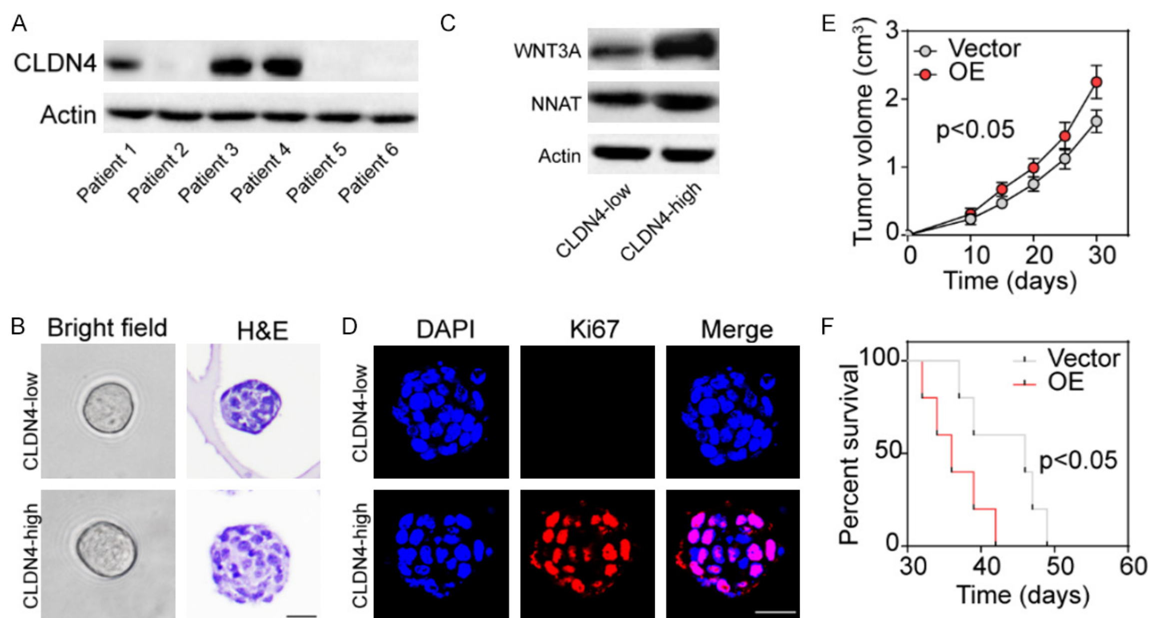


**Figure 4.** CLDN4 facilitates glioma growth *in vivo*. A. Western blotting for CLDN4 in primary glioma cells derived from 6 patients. B. Representative images of PDOs derived from CLDN4-high and -low glioma tissues; scale bar =50 μm. C. Western blotting for Wnt3A and NNAT in PDOs derived from CLDN4-high and -low glioma tissues. D. Representative images for immunofluorescence staining for Ki67 in PDOs derived from CLDN4-high and -low glioma tissues; scale bar =50 μm. E. Tumor volumes of subcutaneous tumors formed by LN229 cells transfected with empty vector or CLDN4-overexpression vector (n=5 in each group). F. Kaplan-Meier survival curve for mice with tumors formed by LN229 cells transfected with empty vector or CLDN4-overexpression vector (n=5 in each group).

primary glioma cells derived from patients exhibited a wide range of CLDN4 expression levels. Subsequently, we established PDOs using glioma tissues with high and low CLDN4 expression levels (**Figure 4B**) and used western blotting to verify that the protein levels of NNAT and Wnt3Awere significantly increased (**Figure 4C**). We also found that the expression levels of Ki67, a marker of cell proliferation, was significantly higher in the CLDN4-high tumors as compared to the CLDN4-low tumors (**Figure 4D**). Furthermore, to explore the influence of CLDN4 on tumor growth *in vivo*, we subcutaneously injected one set of mice with LN229 transfected to overexpress CLDN4, while another set of mice was injected with LN229 transfected with the empty vector. The tumor volumes and survival status of the mice were recorded daily. We found that the LN229 cells overexpressing CLND4 formed tumors that grew much more rapidly than those formed by the cells transfected with the empty vector (**Figure 4E**). The mice injected with the CLDN4-overexpressing LN229 cells had shorter survival times than those injected with the LN229 cells transfected with the empty vector (**Figure 4F**). Together, those experiments indicate that CLDN4 plays an essential role in promoting NNAT/Wnt signaling and stimulating the growth of gliomas, which ultimately results in poor prognoses for patients.

## Discussion

Since many claudins show differential expression patterns in tumor tissues, many studies have investigated the functional relevance of claudins in the growth and progression of tumors. Here, we have identified CLDN4 as a crucial regulator of aggressive traits in glioma cells. In addition, we have shown that high CLDN4 levels are associated with a greater tumor burden in glioma patients. Our findings may be instrumental in gaining a comprehensive understanding of the complexity of glioma progression.

Evidence shows that the altered expression of certain claudin isoforms can significantly affect tumor behavior [18]. Of the claudin multigene family, CLDN4 is one of the most closely studied genes. The upregulation of CLDN4 expression is commonly seen in most epithelial neo-

plasms, where it may be pro-oncogenic or tumor-suppressive, depending on the cellular context [19]. In breast cancer, for instance, CLDN4 expression was positively correlated with tumor grade and could be useful as a prognostic factor [20]. In addition, CLDN4 overexpression triggered increased cell mobility and invasiveness in ovarian cancers, which may be attributed to the activation of MMP proteins [21]. Conversely, Ohtani et al. have reported that low CLDN4 levels were associated with poor differentiation and increased metastatic dissemination in gastric cancers [22]. Although CLDN4 expression has been studied in many tumors, few investigations have analyzed the role of this protein in gliomas. Our study suggests that increased expression levels of CLDN4 in gliomas are associated with poor prognoses. We further demonstrate that CLDN4-overexpressing cells had higher proliferative and migratory capacities which could fuel the rapid growth of gliomas.

Our experiments indicate that NNAT, which is a highly conserved proteolipid involved in brain development, mediates the effects of CLDN4 on glioma growth and progression. The *NNAT* gene is maternally imprinted and changes in the methylation status of the maternal allele may lead to a loss of the imprinting, and therefore, affect cellular functions [23]. A growing body of literature has linked changes in the NNAT expression levels to neurological disorders, diabetes, and even carcinogenesis [24]. Changes in the NNAT expression levels are common in pulmonary adenocarcinomas and could be useful as a prognostic marker [25]. In addition, NNAT overexpression in glioblastoma stem cells is associated with increased cellular proliferation [26]. Our study has shown that increases in the expression levels of NNAT facilitate glioma cell migration and colony formation and that silencing this gene reverses these effects. We have also shown that the tumor-promoting effects of CLDN4 are mediated by NNAT, as the cells overexpressing CLDN4 also have increased expression levels of NNAT. We further show that Wnt signaling acts downstream of the CLDN4/NNAT axis to affect the malignant behaviors of glioma cells. The Wnt signaling pathway is an evolutionarily conserved pathway involved in modulating diverse physiological processes such as proliferation, differentiation, apoptosis, and tissue homeostasis [27]. Dysregulated components of the Wnt signaling pathway are often key players in tumorigenesis and have been investigated as therapeutic targets for many cancers [28]. Previous work on ovarian cancer has shown that the inactivation of the Wnt/β-catenin axis was blocked by the overexpression of CLDN4, hinting that the Wnt signaling pathway was likely to be involved in the pro-oncogenic effects of CLDN4 [29]. Our findings demonstrate that the CLDN4/NNAT axis transmits signals through Wnt3A to induce malignant behavior in glioma cells; this effect could be reversed by the administration of the Wnt inhibitor, pyrvinium pamoate. We have also shown that in xenograft murine models, CLDN4 overexpression resulted in rapid tumor growth and decreased the survival of the mice.

Based on the above results, our study has established the importance of CLDN4 in glioma progression. Firstly, our analysis of data from TCGA database revealed that high CLDN4 levels were associated with lower overall survival times in glioma patients. Secondly, we have proven that glioma cells overexpressing CLDN4 have increased proliferative and metastatic ability as compared to cells that do not overexpress CLDN4. Thirdly, our work has revealed that CLDN4 drives glioma progression through the NNAT/Wnt signaling pathway. Fourthly, we have further validated the role of CLDN4 in the progression of gliomas using PDOs and xenograft models.

## Conclusion

In summary, our findings demonstrate that CLDN4 can enhance the malignancy of glioma cells via the NNAT/Wnt signaling pathway. Targeting CLDN4 may be useful in developing new treatments against gliomas.

## Disclosure of conflict of interest

None.

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## References

[1] Chen R, Smith-Cohn M, Cohen AL and Colman H. Glioma subclassifications and their clinical

significance. Neurotherapeutics 2017; 14: 284-297.
[2] Lapointe S, Perry A and Butowski NA. Primary brain tumours in adults. Lancet 2018; 392: 432-446.
[3] Weller M, Wick W, Aldape K, Brada M, Berger M, Pfister SM, Nishikawa R, Rosenthal M, Wen PY, Stupp R and Reifenberger G. Glioma. Nat Rev Dis Primers 2015; 1: 15017.
[4] Zihni C, Mills C, Matter K and Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. Nat Rev Mol Cell Biol 2016; 17: 564-580.
[5] Kyuno D, Takasawa A, Kikuchi S, Takemasa I, Osanai M and Kojima T. Role of tight junctions in the epithelial-to-mesenchymal transition of cancer cells. Biochim Biophys Acta Biomembr 2021; 1863: 183503.
[6] Martin TA, Mason MD and Jiang WG. Tight junctions in cancer metastasis. Front Biosci (Landmark Ed) 2011; 16: 898-936.
[7] Günzel D and Yu AS. Claudins and the modulation of tight junction permeability. Physiol Rev 2013; 93: 525-569.
[8] Tsukita S, Tanaka H and Tamura A. The claudins: from tight junctions to biological systems. Trends Biochem Sci 2019; 44: 141-152.
[9] Singh AB and Dhawan P. Claudins and cancer: fall of the soldiers entrusted to protect the gate and keep the barrier intact. Semin Cell Dev Biol 2015; 42: 58-65.
[10] Osanai M, Takasawa A, Murata M and Sawada N. Claudins in cancer: bench to bedside. Pflugers Arch 2017; 469: 55-67.
[11] Kumar B, Ahmad R, Giannico GA, Zent R, Talmon GA, Harris RC, Clark PE, Lokeshwar V, Dhawan P and Singh AB. Claudin-2 inhibits renal clear cell carcinoma progression by inhibiting YAP-activation. J Exp Clin Cancer Res 2021; 40: 77.
[12] Katsushima K, Shinjo K, Natsume A, Ohka F, Fujii M, Osada H, Sekido Y and Kondo Y. Contribution of microRNA-1275 to Claudin11 protein suppression via a polycomb-mediated silencing mechanism in human glioma stem-like cells. J Biol Chem 2012; 287: 27396-27406.
[13] Neesse A, Griesmann H, Gress TM and Michl P. Claudin-4 as therapeutic target in cancer. Arch Biochem Biophys 2012; 524: 64-70.
[14] Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Löhr M, Leder G, Iwamura T, Adler G and Gress TM. Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res 2003; 63: 6265-6271.
[15] Luo J, Wang H, Chen H, Gan G and Zheng Y. CLDN4 silencing promotes proliferation and reduces chemotherapy sensitivity of gastric cancer cells through activation of the PI3K/Akt signalling pathway. Exp Physiol 2020; 105: 979-988.
[16] Golebiewska A, Hau AC, Oudin A, Stieber D, Yabo YA, Baus V, Barthelemy V, Klein E, Bougnaud S, Keunen O, Wantz M, Michelucci A, Neirinckx V, Muller A, Kaoma T, Nazarov PV, Azuaje F, De Falco A, Flies B, Richart L, Poovathingal S, Arns T, Grzyb K, Mock A, Herold-Mende C, Steino A, Brown D, May P, Miletic H, Malta TM, Noushmehr H, Kwon YJ, Jahn W, Klink B, Tanner G, Stead LF, Mittelbronn M, Skupin A, Hertel F, Bjerkvig R and Niclou SP. Patient-derived organoids and orthotopic xenografts of primary and recurrent gliomas represent relevant patient avatars for precision oncology. Acta Neuropathol 2020; 140: 919-949.
[17] Nass N, Walter S, Jechorek D, Weissenborn C, Ignatov A, Haybaeck J, Sel S and Kalinski T. High neuronatin (NNAT) expression is associated with poor outcome in breast cancer. Virchows Arch 2017; 471: 23-30.
[18] Kojima T, Kyuno D and Sawada N. Targeting claudin-4 in human pancreatic cancer. Expert Opin Ther Targets 2012; 16: 881-887.
[19] Neesse A, Griesmann H, Gress TM and Michl P. Claudin-4 as therapeutic target in cancer. Arch Biochem Biophys 2012; 524: 64-70.
[20] Lanigan F, McKiernan E, Brennan DJ, Hegarty S, Millikan RC, McBryan J, Jirstrom K, Landberg G, Martin F, Duffy MJ and Gallagher WM. Increased claudin-4 expression is associated with poor prognosis and high tumour grade in breast cancer. Int J Cancer 2009; 124: 2088-2097.
[21] Agarwal R, D'Souza T and Morin PJ. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. Cancer Res 2005; 65: 7378-7385.
[22] Ohtani S, Terashima M, Satoh J, Soeta N, Saze Z, Kashimura S, Ohsuka F, Hoshino Y, Kogure M and Gotoh M. Expression of tight-junction-associated proteins in human gastric cancer: downregulation of claudin-4 correlates with tumor aggressiveness and survival. Gastric Cancer 2009; 12: 43-51.
[23] Pitale PM, Howse W and Gorbatyuk M. Neuronatin protein in health and disease. J Cell Physiol 2017; 232: 477-481.
[24] Joseph RM. Neuronatin gene: imprinted and misfolded: studies in Lafora disease, diabetes and cancer may implicate NNAT-aggregates as a common downstream participant in neuronal loss. Genomics 2014; 103: 183-188.
[25] Uchihara T, Okubo C, Tanaka R, Minami Y, Inadome Y, Iijima T, Morishita Y, Fujita J and Noguchi M. Neuronatin expression and its clin-

icopathological significance in pulmonary non-small cell carcinoma. J Thorac Oncol 2007; 2: 796-801.
[26] Xu DS, Yang C, Proescholdt M, Bründl E, Brawanski A, Fang X, Lee CS, Weil RJ, Zhuang Z and Lonser RR. Neuronatin in a subset of glioblastoma multiforme tumor progenitor cells is associated with increased cell proliferation and shorter patient survival. PLoS One 2012; 7: e37811.
[27] Nusse R and Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 2017; 169: 985-999.
[28] Zhang Y and Wang X. Targeting the Wnt/β-catenin signaling pathway in cancer. J Hematol Oncol 2020; 13: 165.
[29] Kuang L and Li L. E74-like factor 3 suppresses microRNA-485-5p transcription to trigger growth and metastasis of ovarian cancer cells with the involvement of CLDN4/Wnt/β-catenin axis. Saudi J Biol Sci 2021; 28: 4137-4146.

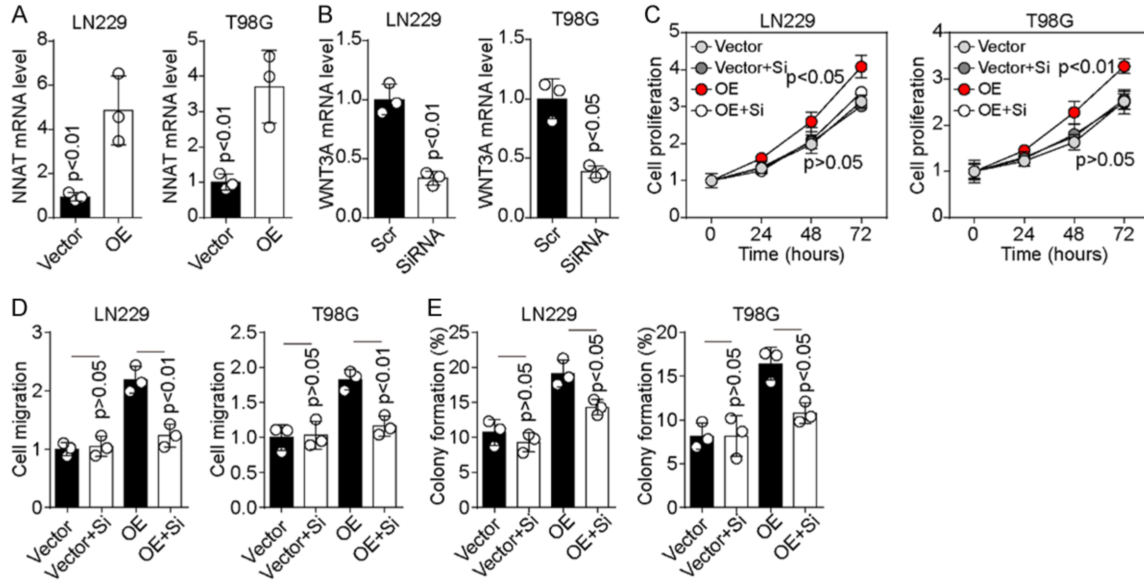


**Figure S1.** A. mRNA expression of NNAT in vector and CLDN4 overexpressed LN229/T98G. B. mRNA expression of Wnt3A in LN229/T98G cells, treated with scramble or Wnt3A siRNAs. C. Cell proliferation of vector and CLDN4 overexpressed LN229/T98G cells, treated with scramble or Wnt3A siRNAs. LN229, vector vs vector + siRNA, P>0.05, OE vs OE + siRNA, P<0.05, 72 hours; T98G, vector vs vector + siRNA, P>0.05, OE vs OE + siRNA, P<0.01, 72 hours; D. Cell migration of vector and CLDN4 overexpressed LN229/T98G cells, treated with scramble or Wnt3A siRNAs. E. Colony formation capability of vector and CLDN4 overexpressed LN229/T98G cells, treated with scramble or Wnt3A siRNAs.