Antibody Design

Introduction

1.1 A Brief Introduction to Glioblastoma

Glioblastoma, also known as glioblastoma multiforme (GBM), is the most common type of brain tumor, taking up to 15% of all primary brain tumors[1]. It is characterized by being highly aggressive towards surrounding normal cells[2], rapid growth and high lethality. The five-year survival rate of GBM patients in the United States is merely 6.8%[3]. In 2021, the World Health Organization classified GBM as grade IV glioma and announced that even with treatment, the median overall survival of patients is 9.9 to 15 months[4]. The glioblastoma can either develop from low-grade benign gliomas[5], or directly occur as grade IV glioma. Symptoms vary based on the location of the tumor, which can affect the function of the brain, for example, speech difficulties, seizures, weakness of the body, and memory loss, but generally with headache, nausea, and vomiting[6]. About 5% of glioma patients have genetic disorders, such as neurofibromatosis, Li–Fraumeni syndrome, and hereditary nonpolyposis colorectal cancer[6]; while the rest of them belong to sporadic GBM. The exact cause of glioblastoma is unknown. However, there are a few identifiable risk factors that might contribute to the occurrence of the cancer, such as being exposed to therapeutic ionizing radiation and vinyl chloride or pesticides; smoking; involving in petroleum refining or production work and rubber manufacturing[6].

1.2 Traditional Treatment of Glioblastoma

Traditional treatment includes surgery, radiotherapy, and chemotherapy. Surgery is an optimal method to decrease the bulkiness of the tumor, leading to the alleviation of symptoms that are brought about by the mass of the tumor. However, unlike solid tumors, GBM has a highly infiltrative nature[7], hence, it is impossible to eliminate all the GBM cells. Radiotherapy and chemotherapeutic drugs are often followed by surgery, in order to damage the DNA of cancer cells, inhibiting the proliferation.

Temozolomide (TMZ) is a FDA-approved, orally administered, acidic alkylating drug, which can cross the blood-brain barrier (BBB) due to its lipophilicity[8]. Temozolomide is a pro-drug, which is very stable at lower pH in the stomach; once drug molecules get absorbed in the small intestine, the hydrolysis interaction happens, breaking the heterocyclic ring, and generating MTIC[8]. MTIC releases methyl diazonium, which adds a methyl group to the O_6 position of guanine of DNA.

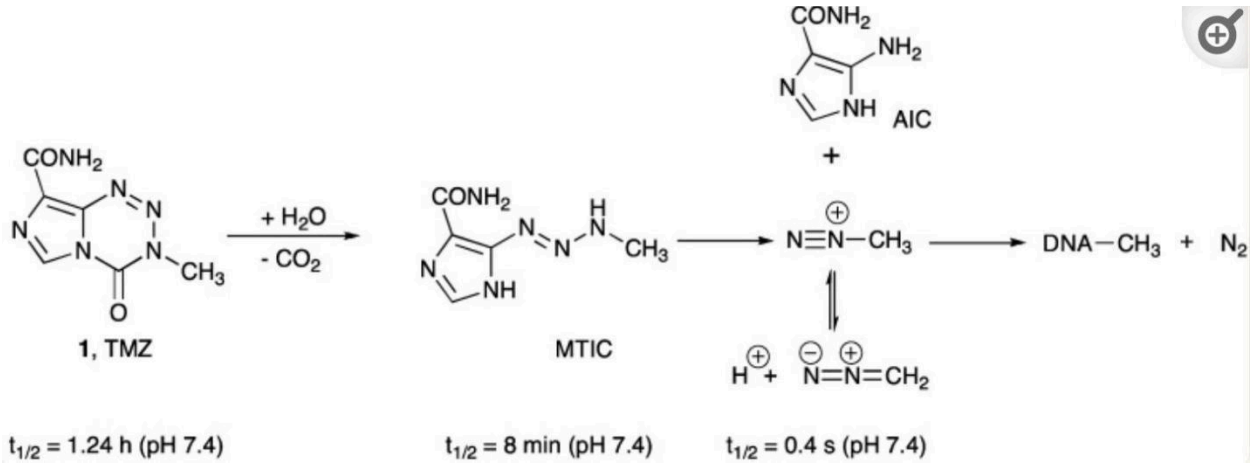

Fig 1. Mechanism of Temozolomide[7]

However, TMZ can trigger severe side effects-myelosuppression with delayed thrombocytopenia, causing interruption of the treatment[8]. Furthermore, it can also decrease the amount of CD4 T cells and B cells[8]. Despite multiple effective methods of treatment, high-grade gliomas can re-occur at the initial position, or disperse to several new positions. This is because GBM cells can narrow themselves, allowing them to migrate through perivascular space around blood vessels and the brain parenchyma space, interacting with brain cells extracellular matrix (ECM)[2].

Furthermore, the function of the O_6-methylguanine-DNA methyltransferase (MGMT) protein is to protect cells from DNA damage[8]. It can eliminate the alkylating agent released by TMZ, thus reducing its effectiveness.

1.3 Glioblastoma Stem Cells

There are two widely accepted hypotheses of how the tumor cells originated. These cells can arise from the stem cells or progenitor cells, where the asymmetrical division process happens and generates transient amplifying cells, which possess a high proliferative capacity[9]. Transient amplifying cells will differentiate into multiple types of cells at last. Secondly, the occurrence of the tumor cells can also be attributed to the reversed differentiated cells, caused by accumulation of mutations[10].

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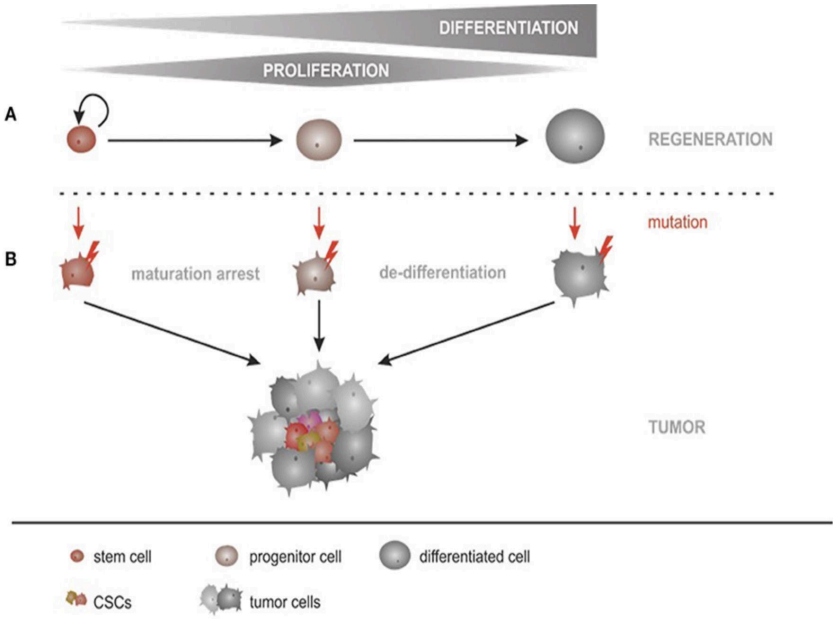

Fig 2. The hypothesis of cancer stem cell initiation[9]

The cancer stem cell hypothesis soon spurred a large amount of studies investigating whether there are stem-like cells in brain tumors. In 2002, [11] proved that NSC-like cells derived from gliomas are not only capable of forming cell colonies, but can also generate cells expressing both neuronal and glial cytoskeletal markers. Galli *et al.*[12] reported that GBM contains neural precursors that share all of the main characteristics with neural stem cells. They also found that these precursors possess unipotent differentiation capacity (can differentiate into astroglial cells) in vivo; while exhibiting multipotent differentiation capacity (can differentiate into neurons, astroglial cells, and oligodendroglial cells) in culture. H. D. *et al*.[13] found that progenitors derived from medulloblastomas and gliomas exhibit the ability of self-renewal and high proliferation; furthermore, these cells can differentiate into both neurons and glia, reflecting the initial tissue where the tumor occurs.

1.4 Vasculogenesis, Angiogenesis process and Glioblastoma Endothelial cells

Angiogenesis is a physiological process that takes place after vasculogenesis. The function of vasculogenesis is to develop vessels from endothelial cells; while through the angiogenesis process, the pre-existing vessels can continuously grow by splitting or sprouting[14]. It is a vital and indispensable process for the formation of granulation tissue[15], tissue growth and development, as well as wound healing[16].

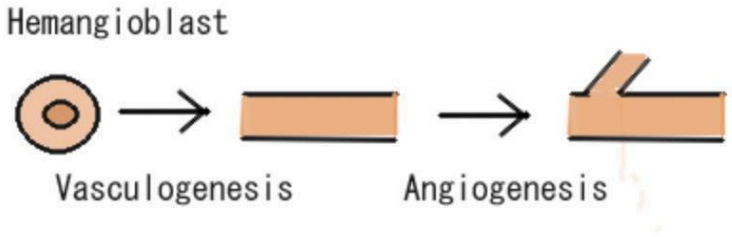

Fig 3. Angiogenesis following Vasculogenesis[15]

However, abnormal angiogenesis can be regarded as the crucial hallmark of cancer[17]. Like normal tissue, tumors also need vascular networks including arteries, veins, and capillaries[18] to obtain nutrients and oxygen, and take away waste that is produced in the rapid proliferation stage. Tissues situated more than 70 μm away from blood vessels tend to experience hypoxia, and if this condition remains unresolved, it can result in cell death through apoptosis[19]. Thus, cancer cells must develop their own blood vessels for survival.

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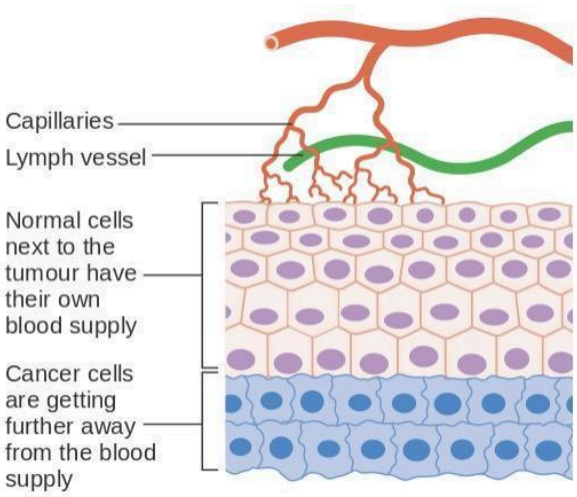

Fig 4. Without angiogenesis, cancer cells can not grow unlimited[15]

Vascular endothelial growth factor (VEGF), as well as the NOTCH signaling pathway in cancer cells, can induce the aberrant growth of blood vessels, which have an irregular shape approaching cancer cells as much as possible.

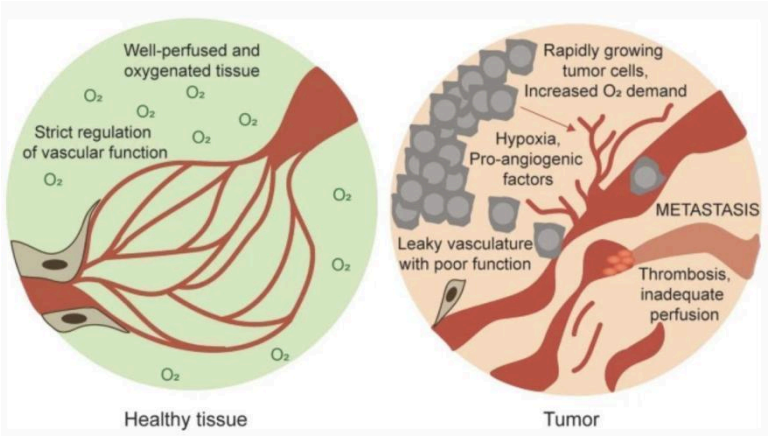

Fig 5. Comparison of vasculature between healthy tissue and tumor[17]

In general, angiogenesis is a required physiological process for a small population of cancer cells to grow into a large tumor. Glioblastoma multiforme (GBM) is distinguished by its abnormal vascular structure, including excessively proliferating endothelial cells, glomerulus-like clusters[7], and blood vessels owning multiple branches but with dead-end[20].

1.5 GBM Cancer Stem Cells Can Differentiate Into GBM Endothelial Cells

In 2020, Wang *et al.*[21] found a subpopulation of endothelial cells separated from glioblastomas that overexpress EGFR as well as chromosome 7, such somatic mutations also exist in GBM cells, suggesting that these endothelial cells are developed from cancer stem cells. In order to validate this promoted hypothesis, they did both *in vitro* and *in vivo* lineage analyses. The result showed that a small colony of stem-like cells has CD133–a kind of glycoprotein on the cell surface, which marks the identification of cancer stem cell[21]. These stem-like cells are multipotent, and able to give rise to tumors; furthermore, there is a subpopulation of CSCs expressing CD144–a protein that is indispensable for the growth of vessels, and has the ability to produce CD133+/CD144+ progenitor cells, eventually differentiate into endothelial cells.

1.6 Notch Signaling Pathway

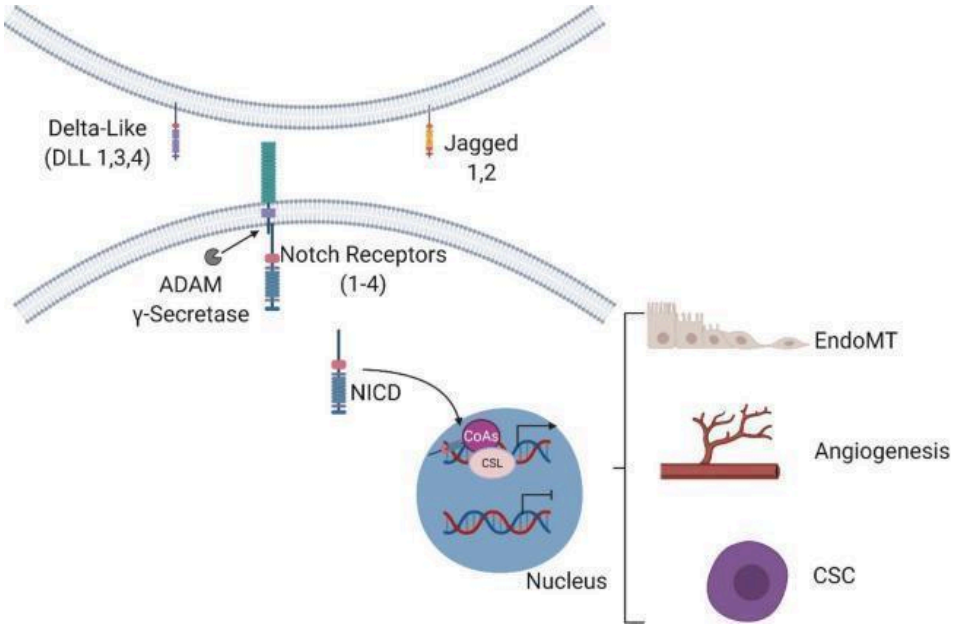

Fig 6. Mechanism of Notch signaling activation and regulation[22]

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Notch signaling is a highly evolutionarily conserved pathways[22] which is widely existent in most animals. The Notch signaling pathway involves multiple biological processes, for example, cell fate determination and regulation of arterial differentiation and angiogenesis[18]; maintenance of stem cells[23]; proliferation and apoptosis[24]. There are four subgroups of the NOTCH receptor family: NOTCH(1~4); and ligands which include five types: Jagged 1, 2; Delta-like ligands 1, 3, and 4[25]. Through non-covalent interaction, the extracellular region ofthe Notch receptor and ligand can bind with each other, thus activating proteolytic cleavage of the intracellular domain of the Notch receptor, leading to the release of Notch receptor intracellular domain(NICD) from cell membrane[18]. And then the NICD translocates to the nucleus to alter the gene expression level, eventually affecting biological function[26].

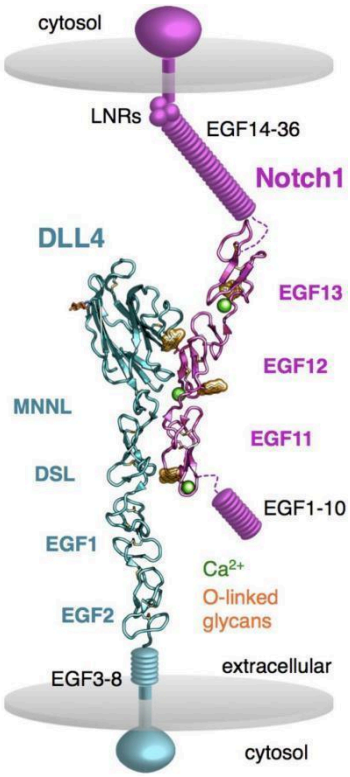

Fig 7.[27] Notch receptor contains 36 repeated EGF domains. Specifically, EGF11 and EGF12[28] involved in the binding interaction with the DLL ligand family

DLL4 is highly expressed in endothelial tip cells, which can display cell migration and proliferation behavior during the proangiogenic process[29]. The Notch-DLL4 signaling pathway has been widely proven to be a vital signal to activate the angiogenesis[30,31]. Another type of ligand-jagged1, overly expressed in cancer cells rather than endothelial cells, now there is a hypothesis claiming that it can act as a communication factor between cancer cells and

tumor-associated endothelial cells (ECs). After binding with a Notch receptor expressed on ECs, endothelial cells can proliferate[18].

Notch signaling pathways can also maintain the survival and self-renewal function of CSCs. It has been proven that using y-secretase inhibitors to block the cleavage of the intracellular domain can decrease the amount of CSCs, and increase apoptosis, thus reducing the resistance to therapies. In general, blocking the Notch signaling pathway can be a promising way to inhibit the proliferation of CSCs as well as the angiogenesis process.

Research Goal

1.1 Specific Aims

Here, we want to design a type of monoclonal antibody that can bind with the Notch1 receptor in the Fv region; while in the Fc domain, we want to introduce a DLL4 mutant with a linker attached to the tail of Fc. DLL4 mutant can compete with normal DLL4 of binding to Notch receptor, thus largely reducing the activation of signaling pathways.

Brain-blood-barrier (BBB) has always been a primary issue for drugs that are designed to target cells in the brain. Although GBM can destroy the completeness of BBB, however, considering the widely existing and indispensable for the vessel growth nature of the Notch signaling pathway, it may lead to severe side effects if most anti-Notch antibodies cannot cross BBB. Thus, we promote two methods of helping the antibody to cross BBB.

2.1 Aim 1

Here, we want to design a type of monoclonal antibody that can bind with the Notch1 receptor in the Fv region first. Considering the molecular weight and the size of the antibody, rather than choose a whole human IgG1 as the framework and only substitute the Fv region, we prefer a vector called pFUSE-Fc1-IgG1 (InvivoGen) which can produce the Fc region (CH2 and CH3 domains) of the human IgG1 heavy chain and the hinge region.

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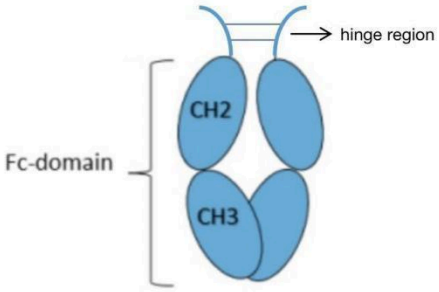

Fig 8. Fc and hinge region of antibody

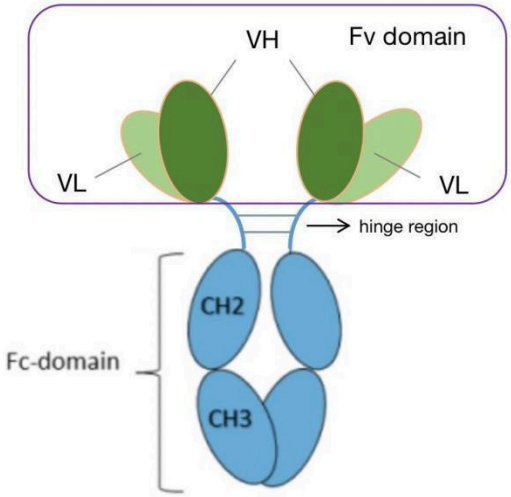

Fig 9. anti-Notch1-Fc1-IgG1 antibody

2.2 Aim 2

In the Fc domain, we want to introduce a DLL4 mutant with a linker attached to the tail of Fc. DLL4 mutant can compete with normal DLL4 of binding to Notch receptor, thus largely reducing the activation of signaling pathways.

The exact domain which involves the interaction between the Notch1 receptor and DLL4 has been clearly identified. Notch1 EGF-like domain 11 and the DLL4 DSL domain can bind together; while such interaction is regulated by the interaction between NOTCH1 EGF-like domain 12 and the MNNL domain of DLL4[32].

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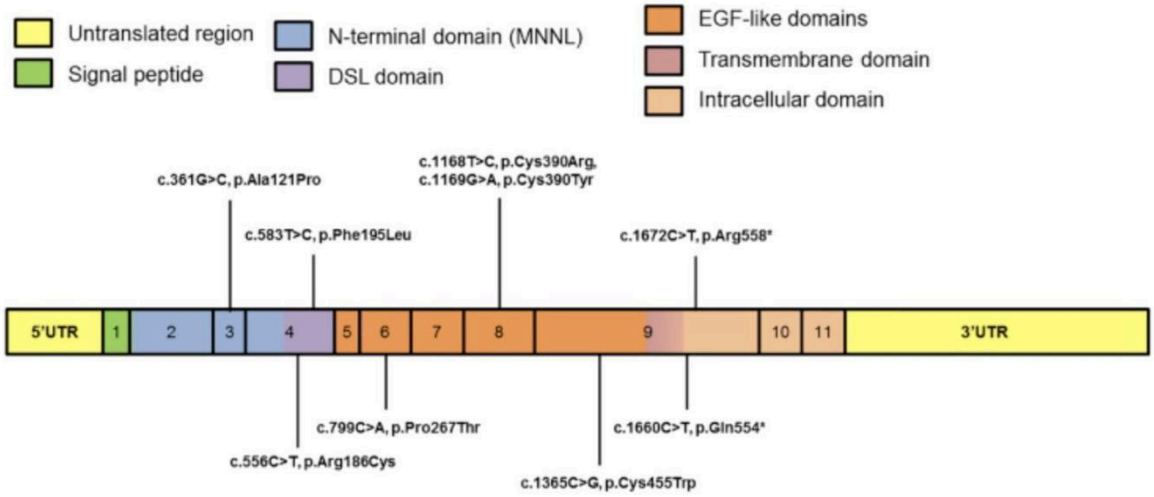

Fig 10. Identified mutations position in all domains of DLL4[32]

Phenylalanine at position 195 is located in the DLL4 DSL domain. Research indicates that the phenylalanine residue plays a crucial role in binding interactions, and substitution with alanine significantly reduces the interaction with Notch1. Thus, we will attach the DLL4 DSL domain with Phe being replaced by Leu at 195 position to the Fc region of the anti-Notch antibody.

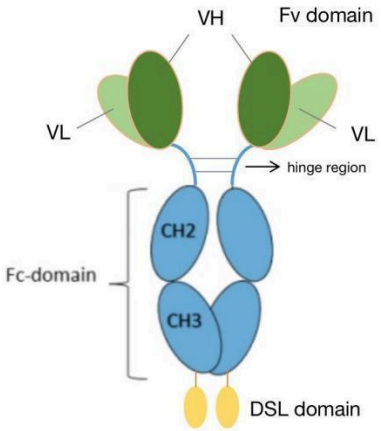

Fig 11. anti-Notch1-Fc1-IgG1-DSL antibody

Antibody Design

2.3 Aim 3

Brain-blood-barrier (BBB) has always been a primary issue for drugs that are designed to target cells in the brain. Although GBM can destroy the completeness of BBB, however, considering the widely existing and indispensable for the vessel growth nature of the Notch signaling pathway, it may lead to severe side effects if most anti-Notch antibodies cannot cross BBB.

Transferrin Receptor(Trf) is highly expressed in the brain, thus, there are lots of studies using the Trf as a target to transport drugs. We aim to use a variable linker, which can attach the anti-Trf construction to the N-terminus of the VH domain. Then the brain endothelial cells can form transcytosis to transport antibodies from the apical membrane to the basolateral membrane, allowing it to enter into the brain.

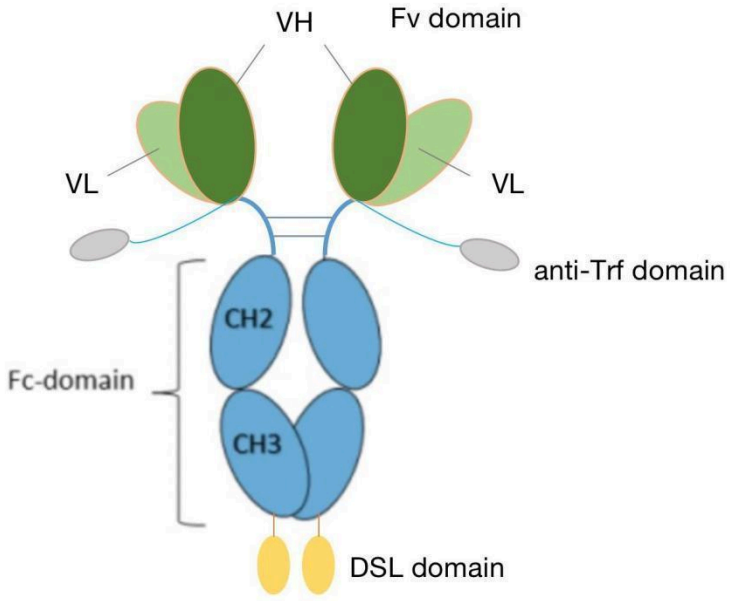

Fig 12. anti-Notch1/Trf-Fc1-IgG1 antibody

Antibody Design

Fasta Sequences

3.1 Aim 1–Construction of Anti-Notch1-Fc1-IgG1 Antibody

3.1.1. Anti-Notch AA Sequence

>N1_E6_scFv_Heavy_Chain_Variable_Domain(123AA)
QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWYNDYAVSVK
SRITINPDTSKNQFSLQLNSVTPEDTAVYYCARGGENWGFGFDYWGQGTLVTVSS

>N1_E6_scFv_Light_Chain_Variable_Domain(111AA)
QSVLTQPPSASGPPGQRVTISCSGSRSNIGAYTVNWYQHLPGTAPKVIIHSNKQRPSGVPDRFSGSKSGT
SASLAITGLQAEDEADYYCQSYDSRLRGWVFGGGTKLTVLG

3.1.2. IgG1 Constant Region AA Sequence

>IgG1_Human_Hinge_Region(23AA)
EPKSCDKTHTCPPCPAPELLGGP

>IgG1_Human_Heavy_Chain_CH2_Region(113AA)
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK

>IgG1_Human_Heavy_Chain_CH3_Region(107AA)
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL
TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPEL

3.1.3. Anti-Notch1-Fc1-IgG1 AA Sequence

>IgG_Combined_Human_Heavy_Chain(VH+Hinge+CH2+CH3: 123 + 23+ 113 + 107= 366AA)
QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWYNDYAVSVK
SRITINPDTSKNQFSLQLNSVTPEDTAVYYCARGGENWGFGFDYWGQGTLVTVSSEPKSCDKTHTCPPC
PAPELLGGPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
MHEALHNHYTQKSLSLSPEL

>N1_E6_scFv_Light_Chain_Variable_Domain(111AA)
QSVLTQPPSASGPPGQRVTISCSGSRSNIGAYTVNWYQHLPGTAPKVIIHSNKQRPSGVPDRFSGSKSGT
SASLAITGLQAEDEADYYCQSYDSRLRGWVFGGGTKLTVLG

Antibody Design

3.2 Aim 2–Construction of Anti-Notch1-Fc1-IgG1-DSL Antibody

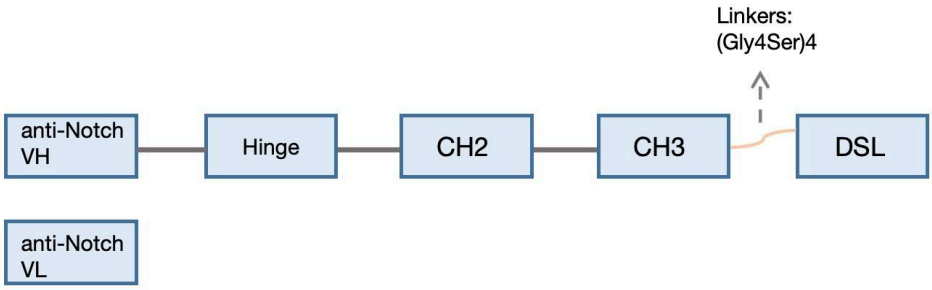

Fig 12. Gene sequence of anti-Notch1-Fc1-IgG1-DSL antibody

>(Gly4Ser)4_Linker(20AA)
GGGGSGGGGSGGGGSGGGGS

>DDL4(Delta-like-protein4)_DSL_Domain_with_F195L_Mutant(45AA)
WT: VICSDNYYGDNCSRLCKKRNDHFGHYVCQPDGNLSCLPGWTGEYC
DSL Mutant: VICSDNYYGDNCSRLCKKRNDHLGHYVCQPDGNLSCLPGWTGEYC

>Whole_Structure_with_DSL_Domain_Heavy_Chain
>(IgG_Combined_Human_Heavy_Chain+Linker+DSL_Domain=343+25+45 AA = 413AA)
QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWYNDYAVSVK
SRITINPDTSKNQFSLQLNSVTPEDTAVYYCARGGENWGFGFDYWGQGTLVTVSSPCPAPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPELGGG
GSGGGGSGGGGSGGGGSGGGGSVICSDNYYGDNCSRLCKKRNDHLGHYVCQPDGNLSCLPGWTGE
YC

>N1_E6_scFv_Light_Chain_Variable_Domain(111AA)
QSVLTQPPSASGPPGQRVTISCSGSRSNIGAYTVNWYQHLPGTAPKVIIHSNKQRPSGVPDRFSGSKSGT
SASLAITGLQAEDEADYYCQSYDSRLRGWVFGGGTKLTVLG

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3.3 Aim 3–Construction of Anti-Notch1/Trf-Fc1-IgG1-DSL Antibody

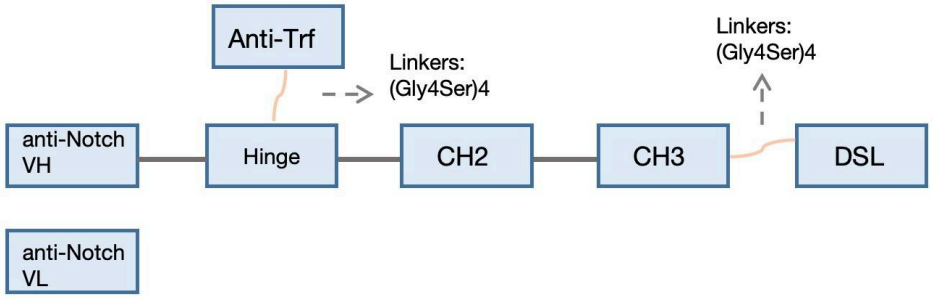

Fig 13. Gene sequence of anti-Notch1/Trf-Fc1-IgG1-DSL antibody

>anti-TRf_Light_Chain(27AA):
RASDNLYSNLADATNLADQHFWGTPLT

>anti-TRf_Heavy_Chain(29AA):
SYWMHEINPTNGRTNYIEKFKSGTRAYHY

Notification: The anti-TRf domain is attached to the hinge region of the heavy chain by the linker

AlphaFold2 Predicted Structure

Please see:
[Anti-Notch Antibody AA Sequence & Protein Structure.md]

Antibody Design

Innovations

1. There are several mature therapies, including Bevacizumab targeting VEGF-A; TMZ; and IR, but unfortunately all of them develop resistance. This proposal focuses on the mechanism of GBM CSCs differentiating into GBM endothelial cells, which can induce angiogenesis to supply the tumor cells, thus we are trying to inhibit the tumor from its initial state.

2. Although the previous study has designed an anti-Notch1 monoclonal antibody[34], it did not modify other regions of the antibody. Due to the high expression of the Notch receptor in the tumor cells, we promote a plausible way to provide four bing sites-two sites for binding with the Notch1 receptor based on the mechanism of antigen-inducing specific antibody; the other two sites are based on the mechanism of receptor-ligand interaction. Thus we largely reduce the activation of the Notch signaling pathway.

3. Considering the severe side effects that anti-Notch antibodies may bring to normal tissue, we designed the motif that can target endothelial cells on BBB.

4. Furthermore, considering the difficulty of biologics entering through BBB, we did not use a whole IgG1 as the framework. We abandoned the CH1 domain and directly fused the Fv region to the hinge region.

Antibody Design

References

1. Young, R. M., Jamshidi, A., Davis, G. & Sherman, J. H. Current trends in the surgical management and treatment of adult glioblastoma. *Ann. Transl. Med.* **3**, 121 (2015).
2. So, J.-S., Kim, H. & Han, K.-S. Mechanisms of Invasion in Glioblastoma: Extracellular Matrix, Ca2+ Signaling, and Glutamate. *Front. Cell. Neurosci.* **15**, 663092 (2021).
3. About Glioblastoma. *National Brain Tumor Society* https://braintumor.org/events/glioblastoma-awareness-day/about-glioblastoma/.
4. Louis, D. N. *et al.* The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. *Neuro-Oncol.* **23**, 1231–1251 (2021).
5. Maher, E. A. *et al.* Malignant glioma: genetics and biology of a grave matter. *Genes Dev.* **15**, 1311–1333 (2001).
6. Alifieris, C. & Trafalis, D. T. Glioblastoma multiforme: Pathogenesis and treatment. *Pharmacol. Ther.* **152**, 63–82 (2015).
7. Ramirez, Y. P., Weatherbee, J. L., Wheelhouse, R. T. & Ross, A. H. Glioblastoma Multiforme Therapy and Mechanisms of Resistance. *Pharmaceuticals* **6**, 1475–1506 (2013).
8. Ortiz, R. *et al.* Temozolomide: An Updated Overview of Resistance Mechanisms, Nanotechnology Advances and Clinical Applications. *Curr. Neuropharmacol.* **19**, 513–537 (2021).
9. Walcher, L. *et al.* Cancer Stem Cells—Origins and Biomarkers: Perspectives for Targeted Personalized Therapies. *Front. Immunol.* **11**, (2020).
10. Blackadar, C. B. Historical review of the causes of cancer. *World J. Clin. Oncol.* **7**, 54–86 (2016).
11. Ignatova, T. N. *et al.* Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* **39**, 193–206 (2002).
12. Galli, R. *et al.* Isolation and Characterization of Tumorigenic, Stem-like Neural Precursors from Human Glioblastoma. *Cancer Res.* **64**, 7011–7021 (2004).
13. Hemmati, H. D. *et al.* Cancerous stem cells can arise from pediatric brain tumors. *Proc. Natl. Acad. Sci.* **100**, 15178–15183 (2003).
14. Oliver, G. Lymphatic vasculature development. *Nat. Rev. Immunol.* **4**, 35–45 (2004).
15. Angiogenesis. *Wikipedia* (2023).
16. Liu, H. *et al.* Epac1 inhibition ameliorates pathological angiogenesis through coordinated activation of Notch and suppression of VEGF signaling. *Sci. Adv.* **6**, eaay3566 (2020).
17. Milosevic, V., Edelmann, R. J., Fosse, J. H., Östman, A. & Akslen, L. A. Molecular Phenotypes of Endothelial Cells in Malignant Tumors. in *Biomarkers of the Tumor Microenvironment* (eds. Akslen, L. A. & Watnick, R. S.) 31–52 (Springer International Publishing, 2022). doi:10.1007/978-3-030-98950-7_3.
18. Akil, A. *et al.* Notch Signaling in Vascular Endothelial Cells, Angiogenesis, and Tumor Progression: An Update and Prospective. *Front. Cell Dev. Biol.* **9**, (2021).
19. Griffin, R. J. International Journal of Radiation Oncology, Biology, Physics. *Int. J. Radiat. Oncol. Biol. Phys.* **66**, 627 (2006).
20. Wen, P. Y. & Kesari, S. Malignant Gliomas in Adults. *N. Engl. J. Med.* **359**, 492–507 (2008).

Antibody Design

21. Wang, R. *et al.* Glioblastoma stem-like cells give rise to tumour endothelium. *Nature* **468**, 829–833 (2010).
22. Lobry, C., Oh, P. & Aifantis, I. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. *J. Exp. Med.* **208**, 1931–1935 (2011).
23. Artavanis-Tsakonas, S. The molecular biology of the Notch locus and the fine tuning of differentiation in Drosophila. *Trends Genet.* **4**, 95–100 (1988).
24. Leong, K. G. & Karsan, A. Recent insights into the role of Notch signaling in tumorigenesis. *Blood* **107**, 2223–2233 (2006).
25. Aquila, G. *et al.* The Notch pathway: a novel therapeutic target for cardiovascular diseases? *Expert Opin. Ther. Targets* **23**, 695–710 (2019).
26. Oswald, F. *et al.* p300 Acts as a Transcriptional Coactivator for Mammalian Notch-1. *Mol. Cell. Biol.* **21**, 7761–7774 (2001).
27. Notch signaling pathway. *Wikipedia* (2023).
28. Rebay, I. *et al.* Specific EGF repeats of Notch mediate interactions with Delta and serrate: Implications for notch as a multifunctional receptor. *Cell* **67**, 687–699 (1991).
29. Siekmann, A. F. & Lawson, N. D. Notch Signalling and the Regulation of Angiogenesis. *Cell Adhes. Migr.* **1**, 104–106 (2007).
30. Benedito, R. *et al.* The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis. *Cell* **137**, 1124–1135 (2009).
31. Blanco, R. & Gerhardt, H. VEGF and Notch in Tip and Stalk Cell Selection. *Cold Spring Harb. Perspect. Med.* **3**, a006569 (2013).
32. Meester, J. A. N. *et al.* Heterozygous Loss-of-Function Mutations in DLL4 Cause Adams-Oliver Syndrome. *Am. J. Hum. Genet.* **97**, 475–482 (2015).

Antibody Design

Amino Acid Sequence Source

Anti-Notch AA Sequence:

Falk R, Falk A, Dyson MR, et al. Generation of anti-Notch antibodies and their application in blocking Notch signaling in neural stem cells. *Methods*. 2012;58(1):69-78. doi:10.1016/j.ymeth.2012.07.008

IgG1 Constant Region AA Sequence:

https://www.uniprot.org/uniprotkb/P01857/entry

IgG1 Human Hinge Region AA Sequence:

Spiteri VA, Doutch J, Rambo RP, Gor J, Dalby PA, Perkins SJ. Solution structure of deglycosylated human IgG1 shows the role of CH2 glycans in its conformation. *Biophys J*. 2021;120(9):1814-1834. doi:10.1016/j.bpj.2021.02.038

DDL4(Delta-like-protein4) DSL Domain WT:

https://www.uniprot.org/uniprotkb/Q9NR61/entry

anti-TRf:

Zhang, Y., Zuchero, J. Y., Atwal, J., Couch, J., Dennis, M. S., Ernst, J. A., Watts, R. J., & Lazar, G. A. (2016). *Anti-transferrin receptor antibodies and methods of use* (WO 2016/081643 A1). World Intellectual Property Organization. https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2016081643. Page 87