

Review

Notch signaling and MicroRNA: The dynamic duo steering between neurogenesis and glioblastomas

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Abstract: Notch signaling is an evolutionary conserved pathway that plays a central role in development and differentiation of eukaryotic cells. It has been well documented that Notch signaling is inevitable for neuronal cell growth and homeostasis. It regulates processes of differentiation from early embryonic stages to fully developed brain. To achieve this streamlined development of neuronal cells, a number of cellular processes are orchestrated by Notch signaling. Abrogated Notch signaling is related to several brain tumors, including glioblastomas. On the other hand, microRNAs are small molecules that play decisive roles in mediating and modulating Notch signaling. This review discusses the crucial role of Notch signaling in the development of the nervous system and how this versatile pathway interplays with microRNAs in glioblastoma. This review sheds light on the interplay between abrogated Notch signaling and miRNAs in the regulation of neuronal differentiation with special focus on miRNAs-mediated regulation of tumorigenesis in glioblastoma. Furthermore, it discusses different aspects of neurogenesis modulated by Notch signaling that could be exploited for the identification of new diagnostic tools and therapies for the treatment of glioblastoma.

Key words: Notch signaling; miRNAs; Glioblastoma; Tumorigenesis; Therapeutics.

Introduction

The alleles of Notch gene were first identified in *Drosophila melanogaster*, which showed a notched phenotype in the wings, in 1917 by T.H. Morgan and it was clear from the outset that it was involved in the most vital cellular developmental processes. It has been found in nearly all metazoan species and the number of its signaling ligands varies from species to species and cell to cell. In mammals, four Notch receptors are recognized, which orchestrate cellular processes ranging from development to homeostasis (1-4). The actions of Notch signaling are mainly intricately by ligand binding to the respective Notch receptor. This ligand-Notch receptor interaction triggers a cascade of molecular signaling that instigate the development and maturity of the stem cells during development and also in adulthood (5, 6). In neuronal stem cells (NSCs), Notch signaling is

tissue-dependent and modulates neural processes including coupling of neuronal growth, differentiation and development of astrocytes (7-9).

This review brings to spotlight the essential role of Notch signaling in the development of the central nervous system (CNS). miRNAs are small molecules with a gene-regulatory role that interact with the Notch signaling cascade during neurogenesis, whose alteration may contribute to glioblastoma. The objectives of this review also include discussing ways in which the applications of the knowledge about this pathway can be extended for discovering unique and therapeutically beneficial tools for glioblastomas treatment.

Notch proteins and ligands

The cascade of Notch signaling requires binding of ligand to the Notch receptor for its activation. Upon

ligand-receptor interaction, the signaling initiates. In mammals, four Notch receptors exist: Notch1, Notch2, Notch3 and Notch4. These are single-pass transmembrane proteins that are located on the surface of the cell in hetero-oligomeric form. Like other transmembrane receptors, Notch receptors also possess an extracellular and an intracellular domain. The extra-cellular domain includes epidermal growth factor (EGF)-like repeats, which number varies among Notch receptors. These repeats play an essential role in receptor-ligand interaction (10, 11). Furthermore, to avoid ligand-free activation of these receptors, three cysteine-rich LIN12/Notch repeats (LNR) are present next to EGF-like repeats. The intracellular domain of Notch receptors consists in six ankyrin/cdc10 repeats (which mediates protein-protein interaction), a RAM23 domain (located N-terminal to ankyrin repeats and facilitates protein-protein interaction) (12), two nuclear localization signals (N1 and N2), a PEST sequence (for negative modulation of protein stability) and a transactivation domain (TAD) (13). Interestingly, Notch1 and Notch3 receptors present a RE/AC (repression/activation) region, located at C-terminal to the ankyrin repeats, required for Notch1 ability to activate and for Notch3 IC's ability to repress a HES promoter. The interaction between the RE/AC region and the ankyrin repeat region provides a basis for interpreting the difference in HES activation between structurally similar Notch receptors. Additionally, each Notch receptor includes three proteolytic cleavage sites: S1, S2 and S3 (14). The Notch receptor is synthesized as single precursor protein; then, the cleavage on these sites facilitates maturation, activation and transportation of the receptor. The cleavage at S1 site at the extracellular domain by the proteolytic activity of furin-like convertase occurs in the Golgi system during intracellular maturation, while cleavages at S2 and S3 sites occur upon ligand-receptor interaction. The ligands of Notch receptors share common structural features with receptor's EGF-like repeats and a distal Cys-rich region called the Delta/Serrate/Lag-2 (DSL) domain. For this reason the ligands are collectively referred to as DSL and include: Delta1/3/4 (Dll) and Jagged1-2 (13).

Notch signaling activation: The pathway's overview

Signal transduction through Notch receptors is triggered upon ligand-receptor interaction, which is followed by a conformational change of the receptor's extracellular portion and detachment of its intracellular portion via proteolytic cleavage (15). The receptor is resistant to proteolytic cleavage prior to ligand binding. The receptor activity at the intracellular domain is monitored by HDAC and a set of co-repressors including NCoR and SMRT (16, 17). These co-repressors act to block gene activation by Notch receptors. Upon cleavage at two sites (S3 and S4) by a presenilin-dependent-secretase complex, the receptor's active form, the Notch intracellular domain (NICD), translocates into the nucleus where it binds to the CSL transcription factor's family (acronym for CBF1/Suppressor of hairless/LAG in humans, *Drosophila* and *C. elegans*, respectively). CSL proteins function as co-repressors of the target genes in the absence of Notch activity, but upon Notch interaction, they transform themselves into co-activa-

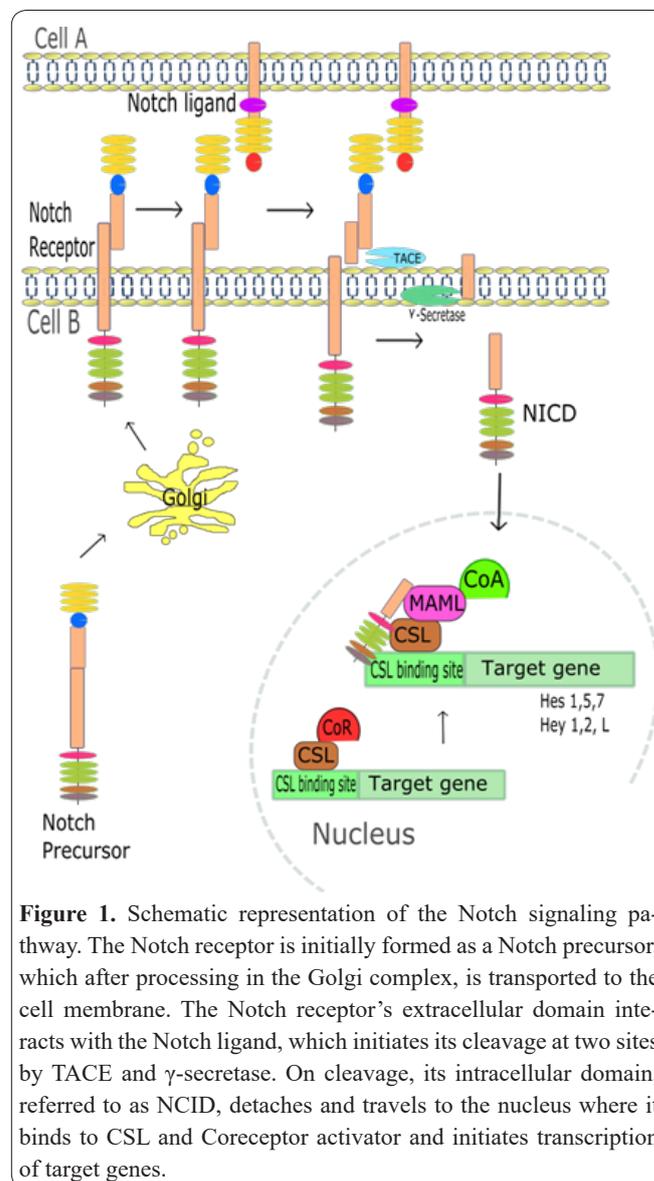


Figure 1. Schematic representation of the Notch signaling pathway. The Notch receptor is initially formed as a Notch precursor, which after processing in the Golgi complex, is transported to the cell membrane. The Notch receptor's extracellular domain interacts with the Notch ligand, which initiates its cleavage at two sites by TACE and γ -secretase. On cleavage, its intracellular domain, referred to as NICD, detaches and travels to the nucleus where it binds to CSL and Coreceptor activator and initiates transcription of target genes.

tors (Figure 1). Two enzymes that actively participate in promoting the transcription of target genes are histone acetyltransferases (HAT) CBP/p300 and PCAF/GCN5, which mediate chromatin relaxation and recruit RNA polymerase II enzyme (3). This results in the expression of Notch targeted genes such as the transcription regulators Hairy Enhancer of Split (HES) and HES-related proteins (Hey). It has been confirmed through studies that the expression of these transcription regulators mediates the stemness and *de-novo* regeneration of (NSCs) (16). Other Notch target genes are c-Myc, cyclin D1, p21, NF- κ B, and SOX2 (18).

Notch signaling in neuronal regulation

Several molecules and mechanisms are involved in the regulation of Notch signaling at the transcriptional and post-transcriptional level. For instance, the Hes gene family curbs gene expression at the transcription level in neuronal cells by maintaining asymmetric cell division through the use of a feedback loop system. At post-transcriptional level, other mechanisms including glycosylation, proteolysis, endocytosis, and degradation modulate Notch signaling (19).

Proneural genes such as ASCL1 and NEUROG2 are demonstrated to drive neurogenesis through the

activation of basic helix loop helix (bHLH) transcription factors (17, 20). Additionally, these genes can also facilitate the regulation of neurogenesis and the differentiation process by inducing the Notch ligands' expression such as Delta like 1 (Dll1) (21). The role of Notch signaling is illustrated in Figure 2. Some studies have also evidenced the proneural genes' involvement with the synthesis of the neuron differentiating genes such as NeuroD. However, lateral Notch signaling can be modulated via the transcriptional feedback loop (22). NICD has positive impact on the expression of transcription factors Hes1 and Hes5 which consequently lead to suppression of proneuronal genes such as Ascl1 and Neurog2. Thus, it brings about inhibition of lateral Notch signaling and the neuronal differentiation process. The promoter complex NICD-RBPj-Hes is primarily involved in repressing differentiation, but without the involvement of this promoter complex, the early differentiation of embryonic NSCs is inconceivable (23, 24). Forward and backward loops created by Hes-mediated oscillations bring the active synthesis and differentiation of Ngn2 and Dll1 mRNA expression to a halt (23, 24).

Imayoshi *et al.* determined through the use of time lapse-imaging analysis of transcription factors Ascl1/Mash1, Hes1, and Olig2 that these factors are expressed in an oscillatory manner by neural progenitor cells. According to them, each factor dominates at different stages, which facilitate fate determination and proliferation of neuronal progenitor cells (NPC). For instance, oscillating Ascl1 expression is necessary for NPC proliferation but its sustained expression is a pre-requisite for fate determination. Altogether, proneural genes promote asymmetric cell division in neuronal cells, facilitating production along with reservation of NSCs via lateral Notch signaling (24).

Notch signaling: Post-translational modifications as modulators in neuronal differentiation and neurogenesis

Post-translational modifications modulate the message transduction through Notch signaling at many

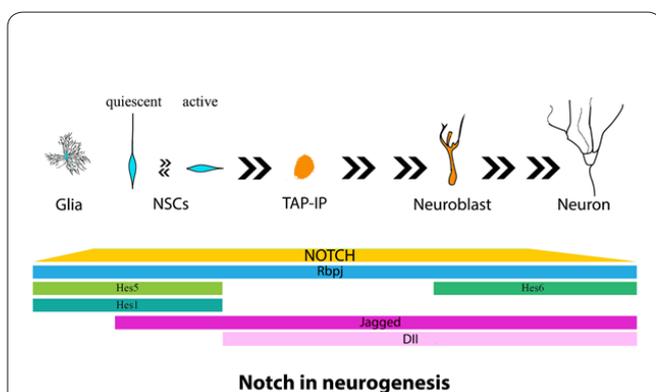


Figure 2. Role of Notch in development of neurons. Notch receptors along with its ligands and neuro-modulator Recombining binding protein suppressor of hairless (RBPJ) are present in almost all stages of neurogenesis. NSCs are being triggered to produce neurons under the influence of Hes family, which is regulated by the Notch signaling. This activation is usually mediated by two Notch ligand families Delta and Jagged. Abbreviation: TAP-IP – Transactivational proteins – Interacting proteins.

levels. For instance, N-O linked glycosylation is a prerequisite for Notch signaling for its functioning during early biogenesis and in secretory pathways (25). However, the machinery behind triggering such changes is currently indistinct. One possible hypothesis described in a review by Fortini *et al.* is that the Fringe family of glycosyltransferases catalyzes the elongation of O-fucose by the addition of N-acetylglucosamines on specific EGF-like repeats of the Notch extracellular domain glycosylation by a N-acetylglucosaminyltransferase (GlcNAC) to O-fructose of the Notch receptor's extracellular domain, disrupting the activation efficacy of Notch to bind to its ligands (26). They further demonstrated that modification of Fringe proteins at the Notch receptor enhances its suitability to bind to a specific receptor in comparison to non-modified Notch receptors. This indicates the fact that post-transcriptional modifications of Notch, including glycosylation, deeply influence the efficacy of the Notch signaling (Figure 3). The findings of Li *et al.* indicated that the binding capacity of Notch is regulated by the presence of O-fucosylation sites, which in *Drosophila* photoreceptor cells promote differentiation defects, such as elevated neuronal apoptosis (27). While loss of function mutations such as fucosylation deletion in mouse and zebrafish are associated with increase in neuronal apoptosis and neuronal plasticity (28).

The second most crucial post-translational modification that greatly impacts the Notch signaling activation is proteolysis and proteolytic cleavage (Figure 3). Specific sites (S1-3) are present on receptors for the proteolytic processing that facilitate maturation and activation of Notch receptors. Notch's pre-processing results in its activation and occurs in Golgi-complex, where the proteolytic cleavage is carried out by a furin-like convertase. However, for neuronal differentiation, cleavage at the S1 site is inhibited by Botch (29). The cleavage of Notch ECD at the S2 and S3 sites is induced by proteases like ADAM10 and γ -secretase which bring about ECD breakage and NICD internalization to the cytoplasm through endocytosis (11). The tight regulation of this process is carried out under the control of genes Numb and Sanpodo (30). This cytoplasmic movement is uni-directional, which through eliciting cytokine release promotes asymmetric neuronal differentiation as well as neurogenesis. One more proteolytic enzyme, α -adapain, assists in internal trafficking of NICD by recruiting the endocytic AP-2 complex to the Numb and consequently promotes neurogenesis and fate-determination of NSCs (31). Studies have highlighted that the duo of AP-2 complex and α -adapain facilitates the coupling of Sanpodo with Numb, which further causes the endocytosis of NICD and regulates the homeostasis of progenitor NSCs. Proteolytic cleavage is required for neuronal differentiation. However, to retain the potential of self-renewal, excessive Notch signaling is a posing threat (30). To deal with the deleterious outcomes of such scenarios, Notch signaling is impeded through the ubiquitylation of the PEST domain of NICD. It is established through various loss of function studies that the presence of the Notch receptor and its ligands are mandatory for the NSCs and a contrary scenario results in neuro-developmental defects, faulty differentiation and aberrant cell transfer in embryonic neuronal cells (28).

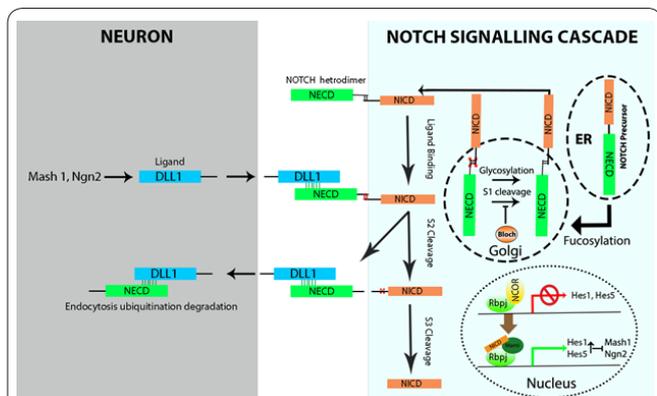


Figure 3. Regulation of Notch signaling is strictly mechanism controlled at several levels. Notch signaling is initiated by the *Ascl1* and *Ngn2*, two transcriptional activators of the bHLH family. Expression of *Ascl1* and *Ngn2* in turn activates expression of the *Dll1* ligand. The *Dll1* ligand activation triggers Notch activation in adjacent cells. The Notch receptor undergoes three stages of cleavage, which are modulated and monitored by glycosylation in the Golgi complex and endoplasmic reticulum. S1 cleavage is accomplished by furin-like protease enzymes that are negatively regulated by *Botch*, and results in the production of two domains: Notch Extracellular membrane (NECD) and Notch intracellular domain (NICD). Binding of NECD to *Dll1* results in the activation of downstream signaling which includes cleavage of NICD by S2. S2 cleavage is mediated by ADAM metalloproteinases. A third cleavage is mediated by γ -secretase that transfers NICD to the nucleus. NICD binds with the CSL complex *Rbpj* in mice that in turn assembles an activator complex containing *Maml*, which promotes the expression of target genes *Hes1* and *Hes5*. In NSCs NICD is being recycled to prevent fate determination.

Notch signaling: Epigenetic modifications as modulators in neuronal differentiation and neurogenesis

Notch interplay with various regulatory pathways of cells is vital for modulation of neuronal cell expression. Both epigenetic alterations and the cross talk between different cellular pathways with Notch assist in NSCs proliferation, development and migration (32, 33). Figure 4 includes the schematic representation of histone modification at different stages of NPC development.

Epigenetic modifications such as DNA methylation, histone modifications and miRNA/LncRNA, have been documented to play an fundamental role in fate determination and differentiation of NSCs (34, 35). The whole scenario is still incomplete; however, compelling evidence has shed light on how these mechanisms play a role in mentoring the Notch signaling pathway (Figure 5).

Notch-driven DNA demethylation is necessary for neurogenesis activation in embryonic cells. Embryogenesis mediation by Notch signaling is dependent on *Hes5* expression and the demethylation of *Glial Cell Missing (GCM)* determines *Hes5* gene transcription (36). *In vivo* studies have demonstrated that the absence of *GCM* leads to abrogation of Notch signaling and impaired neurogenesis; conversely *GCM*'s prolonged expression is associated with differentiation of embryonic neurons from early stage to more advanced stages in a series of transitions. The link between Notch signaling and DNA methylation was elucidated by a study conducted by *Hitoshi et al.* They demonstrated that the

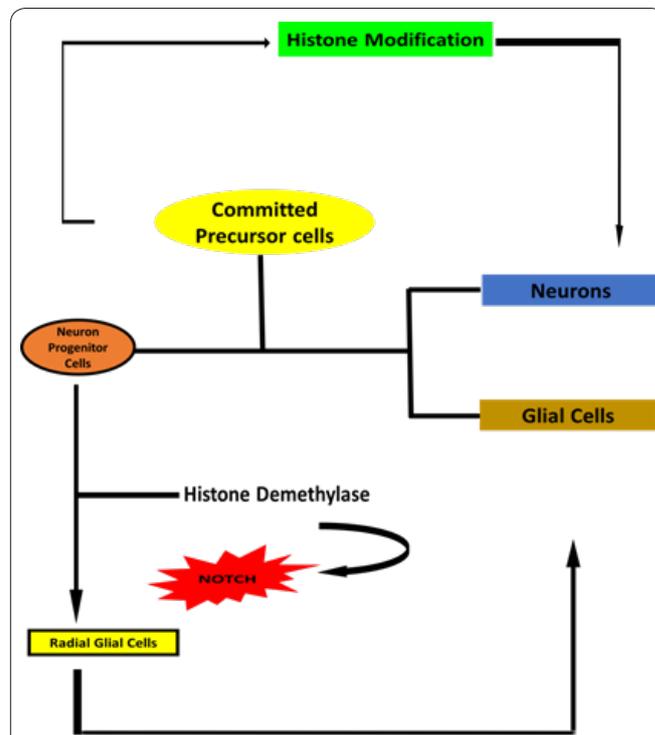


Figure 4. A schematic representation of how histone modification influences neuron progenitor cells to differentiate into either neurons or astrocytes under the influence of histone modification.

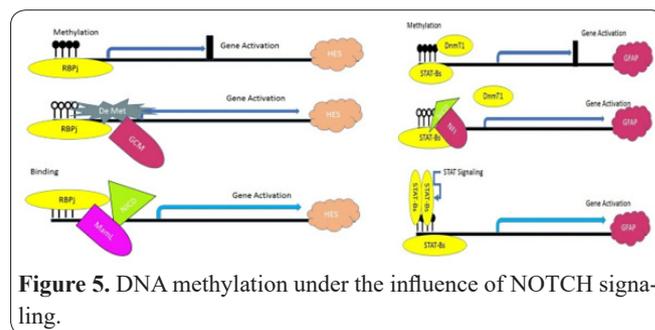


Figure 5. DNA methylation under the influence of NOTCH signaling.

pattern of neuronal differentiation into astrocytes is determined by Notch signaling and DNA methylation interplay (36). During the mid-gestation period, transcription factor *1- α* is secreted by neuronal cells and induces demethylation of *NOTCH1*, *RGMA* and *AKT1* genes which in turn furthers the differentiation of neuronal progenitors and brings about the physiological production of the astrocytes. Notch1 being the mediator of gene's up-regulation also prompts the development of neuronal progeny (37). It has been demonstrated by various evidences that the process of DNA methylation is greatly influenced by the action of Notch1, *Hes1* and *Ngn2* which can in turn generate immense alterations in Notch signaling-mediated DNA methylation (38).

DNA methylation is the key process necessary for gliogenesis under the influence of Notch. NSCs require demethylation of *HES5* via *Gcm* (glial cell missing). Demethylation promotes the activation of the Notch downstream signaling. Upregulation of Notch activity promotes gliogenesis. Activation of Notch in turn promotes isolation of *DnmT1* that induces *STAT* activation, which in turn promotes development of astrocytes.

Histone modifications are the genetic indicators of transcription. Their influence is executed through chromatin modification, assisting in chromosome unwinding and recruitment of transcription factors. The defined

mechanisms, through utilizing which histone modification elicit responses are: phosphorylation, acetylation, ubiquitination and methylation (39). The key enzyme that mediates Notch-dependent transcription activation is Histone acetyltransferase (HAT) which functions through RBPj (40). HAT has also been reported to execute similar modifications in mice, referred as PCAF and GCN5 (41). In the absence of Notch signaling, RBP-J κ /CBF-1 functions as transcription suppressor in humans. CBF1 first recruits HDAC1 and SMRT and forms a repression complex of SMRT/HDAC with another corepressor, SHARP which directly suppresses Hes1 promoter activity and brings about inhibition of Notch mediated signaling (40, 42). HDAC1 role in suppressing Notch signaling in neurogenesis was also documented in zebra fish where suppression of Notch signaling is essential for switching from neuronal proliferation to differentiation (43). Sirt1, a deacetylase, forms a repression complex with LSD1, H4K16 and H3K4 (type of histone demethylases), which control the process of neurogenesis by acting on the Notch-Hes gene (44). The role of histone modifiers in activation and repression of Notch signaling is illustrated in Figure 6.

Interplay of Notch with other pathways

The process of neurogenesis cannot solely be triggered by Notch signaling; therefore, a crosstalk with other cellular pathways is essential for the maintenance of growth and differentiation of neuronal cells.

The bone morphogenetic protein (BMP) pathway prevents glial cell differentiation and brings about the inhibition of neural growth in the primary neurogenesis phase. Upon interacting with the cell surface receptor, the phosphorylation activation of SMAD occurs (45). Like the Notch signaling pathway, the obligatory maintainer of the neuronal stem cells (NSCs), BMP signaling also imposes a particular latency on NSCs (46). Mainly because of sharing of common targets by both signaling pathways including the transcription factor HES family and DNA binding genes' inhibitors (22, 47). Certain idleness might be posed by target gene sharing due to the competition between Hes3 and Hes5 which has been

demonstrated by Hatakeyma and his colleagues' experimentation in neurogenesis. They further revealed that Hes3 is negative regulator of Notch signaling and prevents its premature initiation (48). Rbpj is the chief mediator of the Notch signaling pathway whose expression is a pre-requisite for determination of the neuronal cell's fate. Imayoshi *et al.* induced silencing of Rbpj in model mice and found that Rbpj deletion in embryonic brain led to premature differentiation of all telencephalic progenitor cells (TPCs) into neurons and TPCs depletion. While Rbpj deletion in the adult brain brought about differentiation of all neuronal stem cells into neurons and transit-amplifying cells and depletion of NSCs within three months. (47). These outcomes indicate that signal transduction through the Notch pathway is extremely essential for NSCs maintenance and for regulation of neurogenesis.

The role of Wnt signaling has been explored in the differentiation and programming of neurons (49). The chief element responsible for β -catenin ubiquitination in Wnt signaling is Glycogen synthetase kinase 3 (GSK3) which makes use of axin and other factors responsible for phosphorylation (50). However, in GSK3 absence the Wnt's interaction with frizzled (FZD) receptor, in the presence of Lrp6, is required for its activation. The formation of trimeric complex Wnt/Lrp6/FZD, consequently leads to the prevention of β -catenin phosphorylation and further assists in cytoplasmic translocation of unphosphorylated β -catenin where it induces the transcription of the target genes (51). The interplay between Wnt and Notch signaling is in an agonistic manner where activation of one facilitates the activation of similar transcription factors. Wnt directly brings about the activation of the neuronal genes including Ngn1 and neuroD (genes acting as mediators of differentiation) whereas Notch-targeted Hes5 competes with Ngn1 and neuroD to block neuronal cell differentiation (23).

Among other signaling pathways, FOXO and Hippo interact with Notch signaling at different transcription levels to facilitate growth and differentiation of the neuronal crest. However, due to inadequacy of knowledge on these pathways' explicit role in up-keep and differentiation of NSCs, further efforts are required for better understanding of their involvement (52).

Notch signaling and gliomas

The rate of neurogenesis declines with age. Mechanisms causing this decline have yet to be explored. However, it is considered that the involvement of numerous pathways, metabolic alterations, cell cycle regulation and epigenetic factors are behind this complex process. The association of Notch signaling and aging in neurogenesis has been highlighted in several studies. In aged mice, considerable decline in Notch expression has been reported, which is accompanied by decline of dentate gyrus's NSCs differentiation (53). However, the quiescent NSCs are shown to be revived by kainic-induced seizures in aged mice. In *Drosophila* persistent elevated Notch signaling has negligible effect on old intermediate neuronal progenitors (INPs) while the slightly younger INPs activity is increased by elevated Notch signaling. Therefore, Notch signaling and its downstream target genes play an important role in the

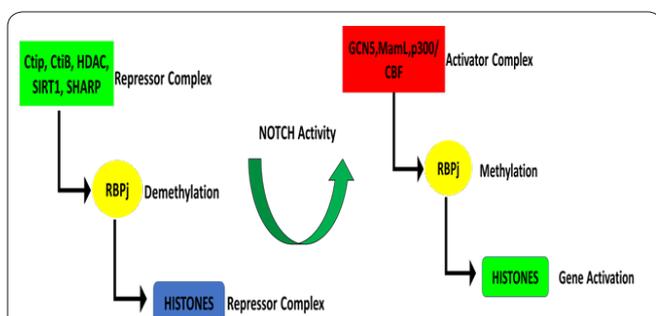


Figure 6. Histone modifiers aid in activation and repression of Notch signaling. In the absence of Notch, the Repressor Complex attenuates RbpJ by the Co-repressor complex consisting of NCOR2/SMRT, SHARP and CtIP/CtBP. These molecules interact with Histone deacetylase HDAC, SirT1 and LSD1 to repress the expression of Notch. While in the presence of Notch, RBPj complexes with Acetyltransferase and P300 and other associated molecules such as GCN5 and PCAF, which leads to active transcription of the target genes.

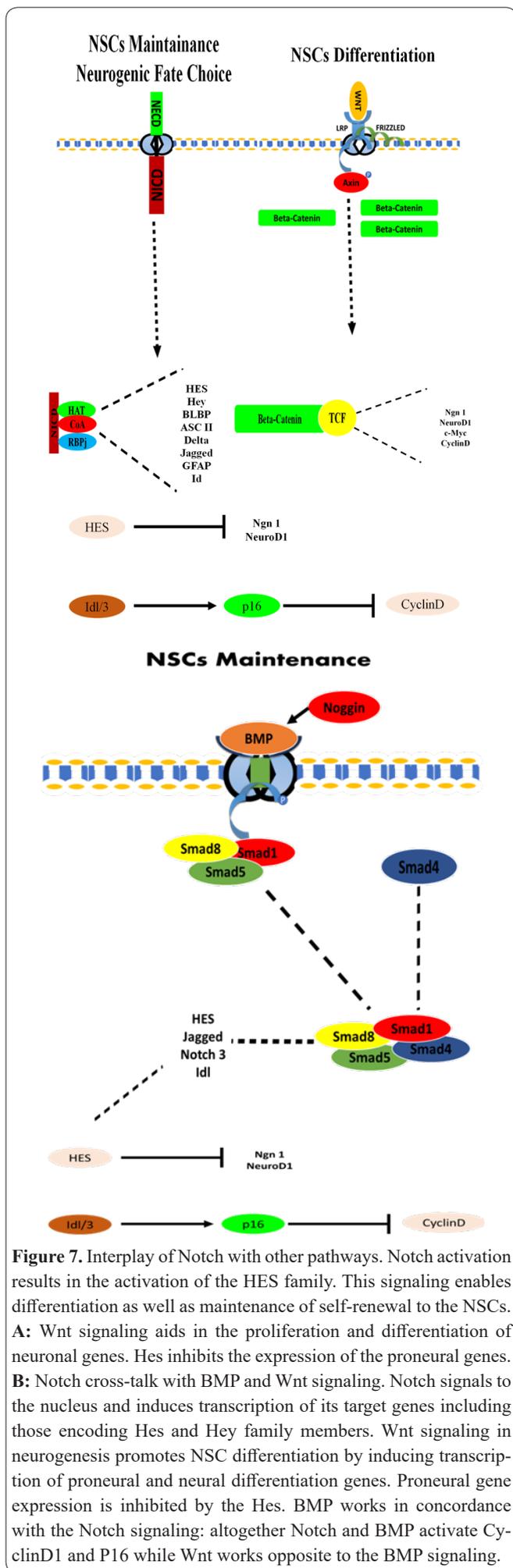


Figure 7. Interplay of Notch with other pathways. Notch activation results in the activation of the HES family. This signaling enables differentiation as well as maintenance of self-renewal to the NSCs. **A:** Wnt signaling aids in the proliferation and differentiation of neuronal genes. Hes inhibits the expression of the proneural genes. **B:** Notch cross-talk with BMP and Wnt signaling. Notch signals to the nucleus and induces transcription of its target genes including those encoding Hes and Hey family members. Wnt signaling in neurogenesis promotes NSC differentiation by inducing transcription of proneural and neural differentiation genes. Proneural gene expression is inhibited by the Hes. BMP works in concordance with the Notch signaling: altogether Notch and BMP activate CyclinD1 and P16 while Wnt works opposite to the BMP signaling.

self-renewal of NSCs (54).

Glioblastoma

The worldwide annual incidence rate of gliomas is high. Among malignant gliomas, the annual incidence rate of astrocytomas and mixed gliomas is 5 per 100,000 individuals (55, 56). Glioma characterization is done on the basis of disease severity and invasiveness into grade I to grade IV. Gliomas, according to classical division, are classified into primary and secondary glioblastomas (57, 58). Its aggressive behavior is because of the involvement of glioma stem cells (GSCs) that not only elevate its tumor potential but also effectively hamper effectively antitumor drugs (59). GSCs hijack the normal growth regulatory mechanism of cells and nurture alongside the neuronal cells (60). Studies have elucidated the role of Notch signaling in the development of GSCs (61). Mutations occurring in the Notch receptor or associated machinery can trigger aberrant Notch signaling that leads to the abnormal growth of the glial cell population (61). Furthermore, to ensure the active proliferation of glioblastoma cells, GSCs induce up-regulation of the Notch receptors after taking control of the cell regulatory mechanism (62). The control also capacitates GSCs to hamper the cell cycle progression, which induces chemotherapy resistance. Studies have also indicated the higher activity of Notch in primary glioblastoma cells as well as resistant glial cells. Furthermore, *in vitro* and *in vivo* studies have demonstrated abrogated Notch signaling as the key element for development of brain tumor and inhibition of Notch signaling leads to cell growth arrest (63). Contrarily, some studies have documented the tumor suppressive role of Notch signaling in gliomas (64). A recent study reported in pro-neural PDGF/P53 that knocking out of Notch signaling resulted in the viability of tumors, which hinted towards Notch role as tumor suppressor (63, 65). Additionally, mutations leading to Notch inactivation have been observed in patients with gliomas and also absence of Notch signaling has been associated with early tumor progression and overall survival. It can be formulated through these findings that the Notch signaling role in gliomas is bi-faceted and requires further explorations.

Glioblastoma multiforme, a grade IV glioma, is very aggressive and has the potential to spread very rapidly. This is the most common form of primary brain tumor, but it is also the most destructive. Glioblastoma multiforme (GBM) has a high rate of malignancy, infiltration and necrosis. GBM current treatment strategies involve surgical excision of tumor, followed by one-year long chemo and radiotherapy sessions. However, drastic side effects and poor survival outcomes accompany this treatment strategy (66). Genetic mutations in glioblastoma regulate a variety of cellular processes that favor tumor growth, development, differentiation, migration, invasion and angiogenesis. Usually, the deregulation of master regulators (including Notch, EGFR, TP53, PTEN and PDGFR) of vital cellular processes gives way to malignancy. Evidence of the fact that tumorous glial cells hijack Notch signaling components came from siRNA-based experimentation. SiRNA-assisted Notch 1 deprivation in glioma cell lines brought about decline in cell growth and elevation in apoptosis (67).

While Notch signaling inhibition resulted in increase in levels of glial fibrillary acidic protein (GFAP) and decrease in vimentin expression that caused increased growth of astrocytes and decline in endo-mesenchymal transition, respectively, in glioma cell lines (68). It is evident from these findings that tumor progression requires maintenance of a stream of undifferentiated glial cells. That Notch1 plays an oncogenic role in gliomas has also been confirmed by xenograft studies in which inhibition of Notch1 and ligand Dll1 caused early demise of mice whereas knocking down of ligand Jagged1 imparted no influence on proliferation and overall survival (67). Notch1 and its paralog Notch2 had opposite effects on the growth and development of subcutaneously engrafted U251 and A172 glioma cell lines. Notch1 knock-down or Notch2 over-expression both had slightly different effects on the pattern of growth of glial cells *in vitro* (69). Under the influence of Notch, Tenascin C (TNC) has been reported to enhance cell migration in gliomas. Notch induced expression of transcription factor RBPj- κ which binds to TNC and lead to proliferation and migration of the GBM cell (70). The consequence of this transformation is increase in GBM based astrocytes number and decreased survival rates of patients (71). The outcome of these studies brings to spotlight the therapeutic as well as diagnostic potential of microRNAs for gliomas.

Notch signaling and implications of MicroRNAs in gliomas

MicroRNAs (miRNAs) are small, non-coding RNAs involved in post-transcriptional processes that inhibit gene translation or induce their destabilization and degradation. In collaboration with Notch signaling, miRNAs coordinate differential growth of neuronal cell as well as their stemness, maintaining regular balance between these biological states. Several miRNAs regulate neuronal differentiation expression via Notch. For instance, Hes1 is targeted by miR-9 and its expression is modulated through a negative feedback loop system (72, 73). It has been reported that miR-9 and its sister stand miR-9* modulate neuronal development by regulating a whole class of Notch receptors (74). Other miRNAs including miR-124 along with miR-9 have been demonstrated to be associated with Notch ligand expression. miR-124, in order to regulate the maintenance of the NSCs, interacts with Jagged-2 (60). Similarly, miR-let-7 promotes the differentiation of glial cell by interacting at the transcription site and targeting Hes-5 (60). Another microRNA, miR-34, by acting on Numbl, has been elucidated to modulate the balance between self-renewal and differentiation of neuronal cell (75).

Aberrent expression of certain miRNAs in neuronal cells leads to development of glioma and glioblastoma. Table 1 and Table 2 consist of lists of miRNAs that play tumor-promoting and tumor-suppressive roles, respectively, by regulating Notch signaling. Wang *et al.* in this regard, demonstrated the crucial involvement of miR-33a in the maintenance of growth and development of GSCs. Confirmation through microarray-based analysis revealed that miR-33a targets phosphodiesterase 8A (PDE8A) and UV radiation resistance-associated gene (UVRAG), which function as negative modulators of

Table 1. List of tumors promoting miRNAs that enhance Notch mediated proliferation.

Tumor promoting miRNA	Target	Reference
miR-524-3p and miR-524-5p	EFGRvIII/c-MYC	(77)
miR-34a	Dll 1	(78)
miR-18a*	Dll3	(79)
miR-21	Mcl-1	(80)
miR-33a	PDE8A/ UVRAG	(76)
miR-92a-3p	Notch domain	(81)
Let-7	Hes5	(60)

Notch signaling along with cAMP-PKA signaling pathway. In particular, antagonizing miR-33a function in GSCs reduced self-renewal and tumor progression in an animal model, whereas over-expression of miR-33a in non-GICs promoted the display of features associated with GICs. This suggests miR-33a as a suitable target for the treatment of glioblastoma (76).

The miR-34 family is the most studied in GBM. Among mRNA targets of the miR-34 family are Notch1 and Notch2. In particular, both miR-34c-3p and miR-34c-5p were found to be down-regulated in GBM and lower levels correlate with a higher glioma grade. This is also supported by the fact that the over-expression of both miRNAs inhibit glioma invasion and only miR-34c-3p increases cell apoptosis and reduces Notch2 expression (46). Other tumor-suppressive miRNAs associated with Notch in GBM and belonging to the miR-34 family are miR-34a and miR-34a-5p. Both miRNAs target Notch1 and Notch2 as well as c-Met, CDK6 and EGFR. In this way they are able to reduce cell cycle progression and cell invasion (82, 83). Moreover, the role of miR-34a as a *trans*-differentiation mediator has been recently reported. Jin Z. *et al.* demonstrated that up-regulation of miR-34a induce a *trans* differentiation of GSCs into vascular endothelial cells (VECs) by targeting Notch1 and Dll1 (78).

Unlike miR-34c, miR-18a* contributes to GSC clonal proliferation and tumorigenicity directly down-regulating Dll3 levels and consequently enhancing Notch1 signaling. This activates ERK, thereby inducing SHH-GLI-NANOG network which is essential for maintaining GSCs and enabling their self-renewal. This finding is indicative of the crucial role for miR-18a* in tumor growth and in controlling the switch between the self-renewing and non-self-renewing states of the GSCs (79).

The presence of low-level persistent expression of the Notch gene is necessary for the prolonged cellular growth of glioblastoma cells. The involvement of Notch signaling and its down-stream signaling molecules in tumorous glial cells' long-term survival is well-documented. Through the application of microarray analysis, Huber *et al.* established a link between glioblastoma cell aggressiveness and over-expression of miR-21. They demonstrated that miR-21-modulated Notch/Deltex pathway is requisite for invasiveness and growth of glioma cells. The culprit that activated Notch canonical signaling is DTX1, which further triggered RTK/PI3K/PKB and the MAPK/ERK mitotic pathways,

hence, leading to the over-expression of anti-apoptotic proteins such as the Mcl-1. Their finding revealed the direct association between miR-21 over-expression and elevated ERK expression which induces cellular growth and stemness of glioblastoma (80).

miR-107, a transcriptional target of p53, is downregulated in glioma samples and cell lines, in particular, p53-mutated U251 and A172 (notably, the miR-107 levels in U87 glioma cells expressing wild-type p53 are higher than p53-mutated U251 and A172). Chen and his colleagues via a lentiviral system approach and GFP assay demonstrated that under the influence of P53, miR-107 facilitates proliferation inhibition and cell cycle arrest at the G0–G1 phase in glioma cells, down regulating Notch2 and CDK6 expression. This points towards miR-107 anti-proliferative activity in brain tumors (84).

Notch signaling is indispensable for angiogenesis, tumor fate determination and survival. The very framework of cancer stemness is delineated by Notch and microRNAs' interaction. Through bioinformatics and biological approaches, Chen and colleagues found that miR-524-5p, which expression is associated with grade and overall survival of gliomas, have direct regulatory control on the expression of two downstream targets of Notch pathway: Jagged1 and Hes1. Levels of both proteins are inversely correlated with miR-524-5p in gliomas. Knocking down of either of these partially phenocopied miR-524-5p re-expression (causing suppressed cell proliferation and invasion both *in vitro* and *in vivo*), whereas forced expression of Jagged-1 or Hes-1 reversed the effects of miR-524-5p on proliferation and invasion of glioma (85)(24).

The key cascade pathway perturbed in gliomas is epidermal growth factor receptor (EGFR) signaling. Recent findings imply the contribution of miR-524-3p and miR-524-5p in suppressing this pathway in gliomas. Both miRNAs are downregulated in the classical subtype of GBM and suppression was associated with EGFR overexpression and EGFRvIII mutation. Indeed, EGFR amplification/mutation can repress the expression of pri-miR-524 by histone modification. The restoration of miR524-3p and miR-524-5p attenuates glioma cell migration, proliferation, and cell cycle, and controls tumor formation *in vivo*. miR524-3p and miR-524-5p inhibited TGF/β, Notch and the Hippo pathway by targeting Smad2, Hes1 and Tead1, respectively.

Notch/TGF beta/Hippo pathways over-expression promotes cellular growth via c-Myc activation that interact with the EGFRvIII promoter region. This finding indicates the significance of miR-524-3p and miR-524-5p as diagnostic markers for glioma development (77). Table 2 consists of list of tumor suppressor miRNAs that positively regulate Notch-mediated apoptosis.

MiR-181c normally reduces cell proliferation, cell invasion and self-renewal abilities through Notch2 downregulation. Unfortunately, miR-181c is downregulated in GBM suggesting a possible role in the malignant behavior in GBM (86).

Two other miRNAs are associated with shorter survival in GBM: miR-148a and miR-31. Both miRNAs were found to be upregulated in tumor tissues and their common target is factor-inhibiting HIF-1 (FIH1), which counteracts HIF-1α, and consequently NICD. Therefore, an upregulation of miR-148a and miR-31 leads to

Table 2. List of tumor suppressor miRNAs that enhance Notch mediated apoptosis.

Tumor suppressor miRNA	Target	Reference
miR-107	CDK6/Notch 2	(23)
miR-524-5p	Jagged 1/Hes1	(24)
miR-199-5p	Hes1	(25)
miR-326	Notch 1	(26)
miR-92a-3p	B-Catenin	(21)

a stabilization of NICD in order to expand and maintain GSCs. Studies revealed that inhibition of these miRNAs prolongs animal survival, depletes the stem cell pool and suppresses tumor growth (87).

The finding of Kefas *et al.* further affirmed the interplay of microRNAs and Notch. They brought to lime-light the crucial interaction between Notch1 and miR-326 in gliomas. In a negative feedback loop system, Notch1 modulated the miR-326 expression by hindering the expression of its targeted genes, thus evidencing the fact that miR-326 delivery could be a potential therapeutic strategy for reducing tumorigenicity of the gliomas (88).

For cancer stem cell growth and development, the importance of expression of miRNAs has been demonstrated in both a murine model of glioma and human glioma cells. However, recent advancement has brought to light that down-regulation of miR-145 enhances the expression of BNIP-3, a BH3-only protein upregulated by HIF1 in hypoxic region of tumors. The authors demonstrated that an overexpression of miR-145 inhibits BNIP-3 transduction, promoting glioma cell death by regulating Notch signaling pathway. In particular, the authors showed that the upregulation of miR-145 and knockdown of BNIP3 decrease protein expression of Notch1, Hes1, and p21 in glioma cells, while miR-145 downregulation and upregulation of BNIP3 increase the Notch1, Hes1, and p21 levels in glioma cells. Lastly, co-transfection of downregulated miR-145 and knockdown of BNIP3 decreased the protein levels of the Notch1-regulated proteins. This suggested that miR-145/BNIP3/Notch axis could represent a novel approach for the eradication of gliomas (89).

Different microRNAs trigger and inhibit the development of glial cells. However, a recent report has shed light on the microRNA that plays a bio-facet role in gliomas. miR-92a-3p was reported to be upregulated in human glioma samples but was less expressed in GSCs compared to glioma cells, suggesting that miR-92a-3p could serve as either an onco-miR or a tumor suppressor in different cell types. Sont *et al.* demonstrated that miR-92a-3p targets CDH1/β-catenin signaling in glioma cells, while Notch1/Akt signaling was the downstream pathway of miR-92a-3p in GSCs. These observations suggest that negative regulation of miR-92a-3p on the migration and invasion ability of glioma cells was partly by blocking the CDH-1/β-catenin signaling pathway, while the reduction of Notch1 by specific siRNA could also inhibit the self-renewal ability of GSCs, suggesting that miR-92a-3p affects the maintenance of stemness of GSCs by down-regulating Notch1 expression (81).

Conclusion

In this review, an outline of the Notch signaling pathway has been connected to neurogenesis and diseases in the brain, concisely depicting the Notch signaling network and its direction at various levels. Notch signaling and its components assume a substantial role not only in control of NSCs movement, but also in structural reorganization of the complex brain framework. In addition to this, Notch signaling regulates the cell cycle, generation of new neurons and monitors the infections that could occur to the CNS. In any case, through the element of Notch in the regulation of NSCs, preservation and differentiation are broadly acknowledged. The accurate molecular mechanism, even in this settled capacity, is not clear and needs further exploration. Notch signaling has been in the spotlight over the past two decades. New findings have related massive involvement of Notch signaling in human brain tumors such as the gliomas. Recent advances in the field of RNA-seq, microarray, high throughput technologies have enabled us to glance at the tiny molecules such as microRNAs in details. Numerous new studies have begun to shed light on the regulatory role of miRNAs in various cellular processes. However, the role of microRNAs in regulating Notch signaling and in gliomas is still bleak. With identification of key miRNAs and Notch signaling elements in gliomas, it is possible to design microRNA-based biomarkers for this complicated anomaly.

Several miRNAs have been reported to be expressed during specific stages of glioma; therefore, miRNAs could be implemented as diagnostic markers for the disease progression. Moreover, few species of miRNAs have been affiliated with the expression of targeted genes and thus play a regulatory role in protein synthesis. Targeting these miRNAs with anti-sense RNAs is a promising approach in devising new therapeutic strategies for cancer.

Over the years natural compounds have emerged as a therapeutic solution for various cancers. Natural compounds are a weapon of choice because of their limited side effects and reduced cytotoxicity compared to modern drug formulations and chemotherapy which produce limitless side effects and high cytotoxicity. The precision and accuracy of the natural compounds is still a debatable question. Natural compounds are highly target specific and have low cytotoxic effect. However, determining the efficacy of natural compound-derived drugs in hampering cellular growth in gliomas still requires many painstaking efforts. Poor survival, late diagnosis, limited therapeutic options are the hindrances that have hampered progress related to devising strategies for gliomas. Despite several advances made in the field of high throughout technology, RNA sequencing, single cell sequencing, chip and molecular biology, and efficient diagnosis of the neuronal malignancies, several stumbling blocks remain that are needed to be overcome in order to devise new therapeutic strategy for different brain related anomalies. Most challenging among them is designing an effective strategy for drug delivery beyond the blood brain barrier (90). Although numerous delivery methods have been tested but so far, no efficient strategy is available that could ensure thorough success. Other than that, the age of the patient, dreadful chemotherapy outcomes and surgical complications for tumors

in deeper regions of brain still need to be addressed. Up to now, the efficacy of extracellular vesicles in delivering drugs for brain malignancies has not been demonstrated experimentally, which theoretically holds potential for overcoming the hurdle of the blood brain barrier. Apart from that, application of nano-therapeutics also seems to present an efficient treatment alternative (91), but natural compounds still present the best option considering minimal cytotoxicity (92, 93). So, development of sustainable drug delivery approach with low toxicity can bring us closer to devising effective therapeutics for brain malignancies. But all efficient treatment strategies are in vain if disease is not timely diagnosed, in which case miRNAs provide a far more valid approach toward early detection of cancer in brain. Unfortunately, the knowledge so far is insufficient to clearly comprehend the whole scenario. So, much more work is required to maximize our understanding and take advantage of the potentials of novel Notch signaling pathway in diagnostic and therapeutic approaches.

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Conflicts of Interest

The authors declare no conflict of interest.

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