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Contradictory Effect of *Notch1* and *Notch2* on Phosphatase and Tensin Homolog and its Influence on Glioblastoma Angiogenesis

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Abstract

Many genes induce angiogenesis in tumors, and among them, *Notch* family genes have received particular attention due to their extensive network of connections with other genes active in this function. Suppression of angiogenic signaling has been studied in various cancers, confirming *Notch*'s fundamental and extensive role. According to studies, four *Notch* genes work independently with many genes such as *vascular endothelial growth factor*, *phosphatase and tensin homolog*, *Phosphoinositide 3-kinase/Akt*, and *matrix metalloproteinases*, and so many other genes, as well as proteins (such as hypoxia-inducible factor-1 alpha) significantly affect tumor angiogenesis. *Notch1* regular activity in a healthy person causes angiogenesis in body tissues, controlled by normal *Notch2* activity. However, in many cases of glioblastoma, whether on patients or tumor xenografts or in vivo models, a mutation in one of these two essential genes or at least one of the genes and proteins that affected by them can cause better angiogenesis in hypoxic conditions and lead to become an invasive tumor. In this review, we examined the contrasting activity of *Notch1* and *Notch2* and the signaling cascade that each generates in the angiogenesis of glioblastoma, the most invasive cancer of the central nervous system.

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Introduction

Glioma is the most malignant type of primary brain tumor that is highly resistant to chemotherapy and other medications. According to the World Health Organization (WHO) classification, glioma with rapid cell proliferation and high resistance to treatment and optimized angiogenesis consist of four histopathological degrees (I-IV), including pilocytic astrocytomas, diffuse astrocytomas, anaplastic astrocytomas, and type IV glioma astrocytoma or glioblastoma multiform (GBM) [1]. Astrocytes are the most abundant type of glial cells in the brain that are multifunctional cells with different central nervous system (CNS) roles and can cause several diseases. They also can control nerve synapses, regulate homeostasis, supply energy to neurons, and recycle neurotransmitters [2–4]. GBM accounts for about 70% of all gliomas [5].

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The current standard of care for patients with GBM includes surgical intervention with maximum possible tumor resection, followed by concomitant radiotherapy (RT) and chemotherapy with temozolomide (TMZ) and then the adjunctive drug TMZ, which is an oral alkylating agent; due to its small size, it is easily absorbed in the intestine and passes through the blood-brain barrier [6,7]. Despite these invasive treatments, patients' maximum survival time is estimated at 14.6 months, with a mortality rate of nearly a hundred percent [8,9]. Tumor angiogenesis is regulated by a complex network of signaling pathways, including *vascular endothelial growth factor (VEGF),* epidermal growth factor receptor (EGFR), peritumoral brain edema (PTBE), P53, and *Notch*. Although not all of the mechanisms involved are known, the *Notch* signaling pathway is critical in tumor angiogenesis [1]. *Notch* signaling plays an important role in regulating many stem cell processes such as proliferation, stem cell maintenance, differentiation during embryonic and adult development, homeostasis of adult regenerative organs, and CNS development [10,11]. In mammals, *Notch* consists of four hetero-oligomer single-pass types I transmembrane receptors (*Notch 1-4)* and five ligands from the Delta-Serrate-Lag family, *including Jagged1 (JAG1)* and *JAG2*, *delta-like 1 (DLL1), DLL3, and DLL4* belong to the Serrate family of ligands. *Notch* receptors are heterodimers with extra- and intracellular functional domains that mediate the target gene's transcription [1]. According to reviewed information and the *Notch* signaling pathway's crucial and contradictory function in inhibiting or inducing angiogenesis, this study aims to determine *Notch1* and *Notch2* in the signaling current that each creates and finally finds a suitable solution to inhibit angiogenesis in GBM or suggest reliable treatment.

1. *Notch* Signaling Expression Pattern in GBM

The *Notch* signaling pathway is disrupted in three-quarters of human GBM, and its growth is suppressed explicitly by inhibiting a receptor in the *Notch* family [12–14]. *Notch1, Notch4, DLL1, DLL2, JAG1, Centromere-binding protein-1 (CBF1), Hairy/enhancer-of-split related with YRPW motif protein-1 (HEY1), HEY2, and hairy and enhancer of split-1 (HES1)* mRNA and protein levels are higher in brain tumor cells than in normal brain tissue, and also with increased *VEGF* and *Phosphorylated protein kinase, strain AK, Thymoma (pAKT)* expressions, and decreased *Phosphatase and tensin homolog* (*PTEN*) levels [15–17]. For example, higher expression of *Achaete-Scute Family BHLH Transcription Factor-1 (ASCL1), DLL1, Notch1, Notch3, Notch4,* and *Hey1* is associated with high-grade glioma and a worse prognosis [18,19]. As a result, the more active *Notch* signaling, the more differentiated and aggressive the tumor phenotype. Some research also suggests that *Notch* activation creates a specific cellular fate, especially distinguishing certain glia types, such as radial glia and astrocytes [20,21].

1.1. Notch1 Signaling Overview

According to studies, among the four *Notch* genes active in GBM angiogenesis, *Notch1* plays the most crucial role. It works in low oxygen conditions concentration with the participation of Hypoxia Inducible Factor (HIF-1α). Suppression of *Notch1* impairs the proliferation and survival of glioma cell lines as well as human gliomas. *Notch1* expression is higher in patients with a chance of survival of more than one year than in less than one year of age. However, *Notch1* overexpression is less associated with overall survival (OS), suggesting a controversial role for *Notch1* in GBM [22,23]. Microglia/macrophages enhance glioma growth by secreting proteolytic enzymes and several angiogenic factors such as matrix metalloproteinases (MMPs), *VEGF,* and affecting nuclear factor kappa B (NF-κB) [24–26]. NF-κB and *MMP*s are involved in tumor cell invasion and tumor angiogenesis, inactivated by *Notch1* inhibition [27]. Besides, the limiting effect of *Notch1* on *PTEN* signaling has been observed [28].

1.2. Notch2 Signaling Overview

Notch2 is known to be a significant prognostic marker in glioma independent of other mutation patterns [29]. The level of *Notch2*

expression in GBM is associated with stem genes *(nestin* and *SRY-Box Transcription Factor-2)*, fate genes (expected outcome of normal development), astrocytes (*vimentin* and *Glial Fibrillary Acidic Protein*), and anti-apoptotic proteins (*BCL6* and BCL-W) but is inversely related to *Oligodendrocyte Transcription Factor-2* (*Olig2), C-type natriuretic peptide (CNP),* and *PLP1* (oligodendrocyte fate) and proapoptotic proteins (apoptosis promoters) such as Bcl-2-associated X protein and Bcl-2-associated transcription factor 1 [30,31]. Positive regulation of *Notch2* expression effectively suppresses cell growth and invasion and induces apoptosis [1]. In a study of the malignant mesothelioma, an invasive tumor of the pleura, pericardium, and peritoneum, *Notch2* negatively regulated *PI3k-Akt* and found that *Notch2* could activate *PTEN,* both of which inhibition of angiogenesis plays a pivotal role [28]. Another study showed that negative *Notch2* regulation induced by siRNA in gastric cancer cells increases the invasive function of tumor cells, enhances the expression and activity of *MMP9*, and increases the phosphorylation of the PI3K pathway, as with growing p-Akt was shown [32].

1.3. Notch3 Signaling Overview

Notch3 is a prognostic factor expressed in the CNS, vascular smooth muscle, and some hematopoietic cells; and enhances cell proliferation, migration, and invasion, which inactivates cell apoptosis [1,33]. Activation of *Notch3* causes invasive glioma formation in the optic nerve but has no confirmed effect on GBM. It has also been observed that *Notch3* increases expression in hypoxic conditions and contributes to significant angiogenesis [34–39]. Some studies suggest *Notch3* has an impact on EGFR gene expression, which could potentiate PI3K function. Also, research shows that *Notch3* plays an essential role in fibroblast-dependent angiogenesis [40,41].

1.4. Notch4 Signaling Overview

High expression of *Notch1* indicates higher differentiation, while increased expression of *Notch4* may indicate a lower degree of differentiation and possibly a tumor with more aggressive function [30]. Not much is known about the cooperation of *Notch4* with other genes affecting glioma, and more studies are needed. Information obtained by Uyttendaele *et al.* [42] shows that among the components of the *DLL4-Notch* pathway, *DLL4* and *Notch4* are expressed explicitly in tumor endothelial cells. This important association and specific morphology showed that *DLL4-Notch4* signaling in endothelial cells plays a vital role in GBM angiogenesis. It has been suggested that *Notch1* and *Notch4* may have similar functions in angiogenesis, regulating, and acting on them in different combinations in different cell types [42]. In summary, the functional role of *Notch1* and *Notch2* compared to *Notch3* and *Notch4* in influencing GBM is better known, scientifically proven, and has a meaningful place in angiogenesis research and the challenging effect of these two genes. Also, their signaling pathway has been studied in various cancers. Numerous articles have examined the promoting or limiting impact of *Notch1* and *Notch2* on cell lines, tumor xenografts, animal models, and human tumor specimens.

2. *Notch* Signaling and Angiogenesis

Blood vessel formation is a dynamic and complicated process that plays a crucial role in health state and disease vulnerability. Delicate balance-dependent angiogenesis is regulated between anti-angiogenic and pro-angiogenic molecules and angiogenesis in tumors. Angiogenesis occurs when pro-angiogenic stimuli are more potent [43,44]. Among the many signaling pathways that affect angiogenesis, the *Notch* signaling pathway is a ligand-receptor cascade that plays a vital role in guiding cellular fate and vascular development and inducing tumor angiogenesis [45,46]. Two key ligands mediate *Notch* paracrine receptors in glioma stem cells (GSCs), *DLL4*, and *JAG1*, expressed in epithelial cells (ECs) [47].

2.1. DLL4

DLL4 is a *Notch1* ligand that plays a vital role in vascular development and is present in active angiogenesis sites. Predictors of tumor progression and survival are indepen-

dent of age, sex, WHO grade, *PTBE*, and expression levels of Ki-67, MGMT, and p53 [48–52]. Li, Z. *et al*. showed that in different ECs classes, hypoxic conditions lead to induction of *DLL4* by HIF-1α [50]. *DLL4* levels in tumors and vascular tissue are considered as predictive markers [53]. *DLL4-Notch* signaling pathway interacts with several molecules and other signaling pathways, including PI3k, EGFR, and *MMP9*, all related to tumor invasion, proliferation, and metastasis. *Notch1*-dependent activity in the *PI3k-Akt* pathway via *DLL4* leads to cell migration and invasive cancer [17,54,55].
According to previous observations, overexpression of *DLL4* in glioma connective tissue reduces vascular density, improves vascular collapse, reduces intra-tumor hypoxia and necrosis, and ultimately prevents tumor growth. In contrast, inactivating *DLL4* causes unproductive angiogenesis (production of a dysfunctional vessels network) with necrosis and hypoxia. *DLL4-Notch* signaling activity in tumors enhances blood vessels' better perfusion (productive vessels), stimulating tumor growth despite the reduced vascular density and improving function within a tumor [56,57]. Besides, the *Notch* pathway also regulates tumor cell differentiation into ECs in several ways [14,58,59].

2.2. JAG1
The *Notch /JAG1* signaling pathway can work directly with other essential pathways such as *MMP9* and *VEGF* to regulate glioma growth and malignancy, which defines patients' physical condition with glioma [60]. Numerous articles indicate the *JAG1* signaling pathway as a modulator of angiogenesis associated with *DLL4*, with *JAG1* somewhat limiting *DLL4* function to keep it out of control. In other words, *JAG1* and *DLL4*, as *Notch1* ligands, together cause normal angiogenesis, and any dysfunction of either causes inefficient angiogenesis and invasive tumor [61].

2.3. HES1
HES1 is a transcription factor and downstream target in the *Notch1* signaling pathway. According to studies, it is located on human arterial ECs, and its significant effect on the regulation and morphologic changes of angiogenesis is confirmed [62]. Tumor growth factor-α (TGF-α) can also regulate *HES1* expression independently of *Notch1* function and introduce *HES1* nuclear import in the presence of ERK1/2 activation. They synergistically promote the growth of glioma cells [63]. One of the critical tasks of *HES1*, which has been mentioned in various studies, is to cooperate in the development of tumor angiogenesis under the control of *Notch1* and inhibit *PTEN* function [64–67]. The importance of *DLL4* and *JAG1* in their complementary function is that the expression of *DLL4* in GBM is limited to endothelial cells. It is significantly more common and severe than *JAG1; DLL4/Notch* angiogenesis's high activity exacerbates GBM. *HES1* transcription factor's role as *Notch1* operating lever in the control and inhibition of *PTEN* was investigated in many studies.

3. Factors That Affect *Notch* Performance

Many molecular components are involved in and affect the *Notch* signaling pathway, the most important of which is hypoxia, affecting *Notch1*/*2* performances through the HIF-1α induction factor. This effect can be inhibitory or inductive.
Another important pathway leading to angiogenesis regulated by *Notch1*/*2* and having significant hypoxia activity is *PI3k-Akt-MMP9,* which is inhibited by the *PTEN* removed on chromosome 10 [68–72]

3.1. Hypoxia Regulate Angiogenesis in GBM
Tumor angiogenesis is essential for tumor growth and progression, and solid tumors often have increased hypoxia, a potent angiogenesis stimulus. Low oxygen levels may be due to structural abnormalities in the tumor vessels or the tumor size, leading to inadequate oxygen delivery. Changes in gene expression may help the tumor adapt to its hypoxic environment. One of the induced genes is HIF1. HIF1 is a heterodimeric protein belonging to the basic helix–loop–helix family of transcription factors. It regulates the expression of many genes involved in tumor progressions, such as *VEGF* and *Notch* [73]. Before the induction of angiogenesis,

cells survive in the tumor mass inside and away from blood vessels in nutrient deficiency conditions and inadequate oxygen supply. The association between hypoxic conditions and the *Notch* pathway in GMB has been reported in several studies [68–72,74,75]. Under hypoxic conditions, the presence of HIF-1α increases the stability of the *Notch* protein and the physical interaction between the two proteins [68]. Hypoxia is one of the hallmarks of GBM, and *Notch* signaling works best with hypoxia. Five hypoxia markers (HIF-1α/PGK1/*VEGF*/CA9/OPN) have been identified as the best predictors of *Notch1*, *DLL1, HES1, HES6, HEY1,* and *HEY2* induction. Also, under hypoxia, GSCs express several *Notch*-related genes *(Notch1, Notch3, DLL1, JAG1, JAG2, HES1, HEY1, HEY2)* and hypoxia-related genes *(HIF-1a, VEGF, LOX,* and *HIG2)* increases [69–71]. HIF-1α induces *Notch* pathway activity and makes GSCs more sensitive to maintenance in hypoxic conditions. Hypoxic conditions by activating *Notch* signaling lead glioblastoma cells to increase colony formation, increase cancer stem cell markers' expression, increase neurosphere production, and malignancy. Various mechanisms are involved in the proper formation of blood vessels observed in these tumors. The germination of capillaries caused by existing blood vessels through endothelial proliferation depends on hypoxia at the tumor center [72]. Hypoxia induction factor, which enhances transcription of *VEGF*, is activated in GBM. Hypoxic tumor cells, especially cell around the necrotic nucleus, release vascular growth factors such as *VEGF*, which stimulates the formation of new blood vessels from existing normal endothelial cells [72]. The researchers also explained that *Notch* activation due to hypoxia could be reversed through targeted *Notch* therapy. Contrary to the role of HIF-1α in *Notch* signaling, the reaction of HIF-2α and *Notch* intracellular domain suppresses *Notch* signaling. HIF-1α and HIF-2α bind competitively to the *Notch* intracellular domain and dynamically regulate *Notch* signaling activation in GSCs depending on different oxygen stresses (concentration changes), and improved treatment opportunities provide oxygen for various strains [74,75].

3.2. Over Expression of PI3k-Akt Signaling Cause Angiogenesis

One of the *Notch1* angiogenesis mechanisms is the induction of *PI3k-Akt* pathway activity via *DLL4*. PI3k/Akt signaling regulates angiogenesis by affecting expressions of *VEGF*, HIF-1α, and *MMP9* [57,76–79].

Activation of PI3k by *Notch1* also stimulates the signaling pathways of *MMP9*, β-catenin, and NF-κB, which increases the migratory, invasiveness, and angiogenic properties of glioma cells [17,77,78]. Inhibition of the PI3K pathway not only limits tumor cell growth but also inhibits tumor angiogenesis. Interestingly, the PI3K pathway plays an influential role in regulating *VEGF* and *VEGFR* [80]. Fibroblast growth factor receptor (FGFR) modulates several tumor cell processes, including FGF-mediated migration and proliferation. FGFR plays a substantial role in the survival and angiogenesis of glioblastoma cells via the PI3k/AKT/mTOR signaling pathway [81].

3.3. MMP9 Effects On Angiogenesis

MMP9 is a family of related enzymes that destroy the extracellular matrix and are essential factors in facilitating tumor invasion and metastasis [82–84]. *MMP9* is a downstream target for the PI3k/Akt pathway that is crucial in cell proliferation control [77,78]. Under physiological conditions, *MMP9* plays a vital role in tissue repair in connection with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, osteoarthritis, and metastasis [85,86]. *MMP9* is required to maintain normal/healthy tissue structure and epithelial integrity. Abnormal expression and activity of *MMP9* have been reported in pathological conditions, especially in various cancers [85–90].

3.4. PTEN Controls PI3K/AKT Activation

PTEN is a tumor suppressor that neutralizes the PI3K/Akt/mTOR pathway with its lipid phosphatase function. Mutation and methylation of *PTEN* have been detected in at least 60% of GBM [91]. *PTEN* may contribute to gliomagenesis and survival by impairing proliferation, migration, invasion, angiogenesis, stem cell self-renewal, and regulation of other tumor suppressor pathways such as P53,

poorly associated with glioblastoma [91–95]. It has been observed that *Notch1* and *Notch2* have different effects on *PI3k-Akt* signaling with opposed regulation of *PTEN*, which was confirmed by protein and mRNA level analysis. *PTEN* activity can also have a limiting impact on HIF1-α and *VEGF*. Moreover, thereby inhibiting angiogenesis and tumor survival [28,79]. Specifically, in GBM, *PTEN* loss leads to the expression of *VEGFR2* in tumor cells, which may play a role in resistance to angiogenesis inhibitory therapies. A new study also showed that overexpression of *VEGFR2* in tumor cells could induce early GBM resistance to TMZ chemotherapy and anti-angiogenic therapy with bevacizumab [96]. Taken together, it seems that *Notch1* and its signaling pathways such as *PI3k-Akt* and *MMP9* play a significant role in tumor angiogenesis with the help of *VEGF* and HIF1-α factors; admittedly, the presence of hypoxia plays a significant role in exacerbating angiogenesis, and in normoxia, less pro-angiogenic factors will be present. Unlike *Notch1*, *Notch2* has a more substantial role in tumor growth and plays a suppressive role in tumor angiogenesis through *PTEN* induction and AKT dephosphorylation [28]. Defects in the expression or function of *PTEN* are also indirectly associated with anti-angiogenic drug resistance [96].

4. Influence Inhibition of *Notch1* and *Notch2* -Induced Expression On Angiogenesis

Due to *Notch1* and *Notch2* proteins' contradictory function in GBM angiogenesis, regulating their activity and downstream signaling for therapeutic methods has been investigated in many articles. In a single-gene therapy study and multi-gene combinatorial therapy on EGFR, PI3K, *AKT,* and *PTEN* in GBM, Han *et al*. reported that *PTEN* was upregulated by adenoviral-mediated *PTEN* (Ad-*PTEN*),

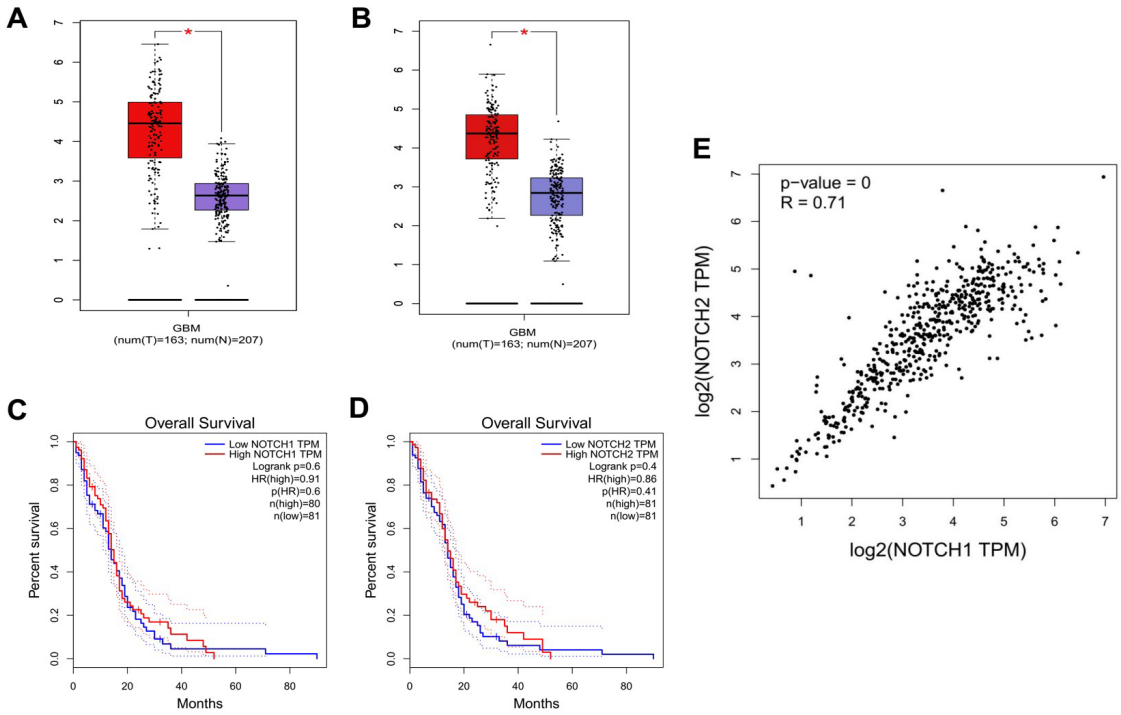

Figure 1. Evaluation of expression, survival, and correlation between *Notch1* and *Notch2* in GBM. **A** and **B**: The expression level of *Notch1* and *Notch2* is shown as a box plot with a significantly higher expression rate in patients with GBM than healthy people. **C** and **D**: The survival chart between these two genes is about 45 months; the survival rate in patients with high expression of these genes is close to zero. **E**: Correlation between two genes has been shown that with increasing expression of each gene, the other gene has also increased expression. It can be said that these two genes are related to each other. The diagrams were drawn using the GEPIA database.

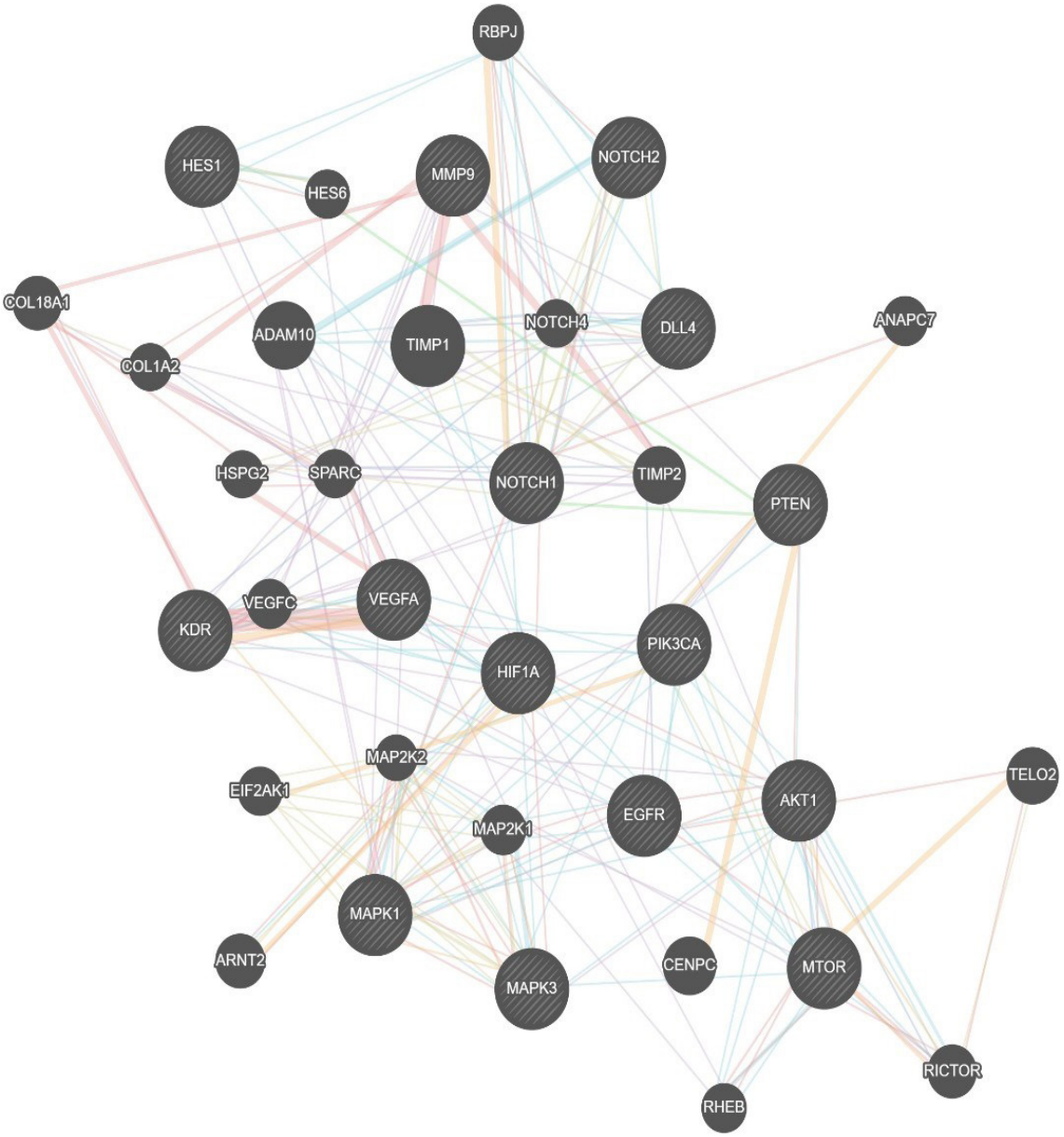

Figure 2. The network drawn using the GeneMANIA database shows the relationship between the upstream and downstream genes *Notch1* and *Notch2*. The circles and the connections between the lines of each gene show the importance of that gene in the network.

and PI3K was suppressed by LY294002 (Figure-1, Figure-2) [95,97,98]. The effect of this combination therapy was evaluated on glioma cell lines (U251 and LN229) and tumor xenograft (U251). Although multi-gene combination therapy is far more effective than selective gene therapy, it still cannot completely inhibit glioma growth, and further studies are suggested [95,97,98].

In another study, researchers used *Notch1* siRNA to reduce *Notch1* function and increase Plasmid-induced *Notch2* expression. *Notch1* siRNA transfer to GSCs suppresses the *Notch1* gene. *Notch1* mRNA level and according to Western blot analysis, *Notch1* protein was significantly reduced in this group compared to the control group [99,100].

Conflict of Interest

The authors declared that they have no conflict of interest.

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