

Differential Expression of Notch Family Members in Astrocytomas and Medulloblastomas

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Abstract Notch signaling pathway plays an integral role in determining cell fates in development. Growing evidence demonstrates that Notch signaling pathway has versatile effects in tumorigenesis depending on the tumor type, grade and stage. Notch signaling pathway is deregulated in some brain tumors. To examine the differential expression of Notch family members (Notch1, 2, 3, 4) in human astrocytomas and medulloblastomas, and to evaluate their roles in the development of both tumor types. Immunohistochemical staining and Western blot analysis were used to detect Notch1, 2, 3, 4 expression in tissue microarray and freshly resected tissue samples of normal brain, astrocytomas and medulloblastomas. Notch family members were not expressed or barely detectable in normal brain tissues. Notch1, 3, 4 were highly expressed but Notch2

was not expressed in astrocytomas. The percentage of immunopositive tumor cells and level of Notch1 expression was increased with tumor grade. In addition, overexpression of Notch2 was detected in medulloblastomas in contrast to low or no expression of Notch1, 3, 4. Differential expression of Notch1, 2, 3, 4 is detected in astrocytomas and medulloblastomas, that may be related to their different roles playing in the development of brain tumors.

Keywords Astrocytomas · Differential expression · Medulloblastomas · Notch family members

Abbreviations

AST	astrocytomas
CBF	C promoter binding factor
CIR	CBF-interacting repressor
CSL	CBF-1/RBPJk-Su (H)-LAG1
EGL	external granule layer
GNPs	granule neuron precursors
HES	hairy-enhancer of split
HERP	HES-related repressor proteins
MAML-1	mastermind-like 1
MB	medulloblastomas
NICD	notch intracellular domain
pCAF	P300/CBP-associated factor
PEST	proline-glutamate-serine-threonine-rich
PNET	primitive neuroectodermal tumors
RAM	RBP-JK-associated molecule
SAGE	serial analysis of gene expression
SMRT	silencing mediator of retinoid and thyroid receptors
TAD	transcription transactivation domain
T-ALLs	T cell acute lymphoblastic leukemias

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Introduction

Notch signaling pathway, originally discovered in *Drosophila*, participates in a wide variety of cellular processes including maintenance of stem cell self-renewal, proliferation, specification of cell fate or differentiation, and apoptosis [1, 2].

Notch genes encode a family of receptors that mediate short-range signaling events. In mammals, there are four Notch receptors, Notch1, 2, 3, 4, as well as five ligands, Delta-like 1, 3, 4 and Jagged 1 and 2. Notch receptor is a single transmembrane protein containing distinct structural domains. The extracellular domain contains many EGF repeats and three cysteine rich Notch/Lin 12 repeats. The amino-terminal EGF-like repeats bind with the ligands. The intracellular domain has a RAM (RBP-JK-associated molecule) domain, six ankyrin repeats, two nuclear-localization signals, a transcription transactivation domain (TAD) and a proline-glutamate-serine-threonine-rich (PEST) domain. The structure of four Notch receptors is highly homologous. There are only some differences in the number of EGF repeats in extracellular domain and the presence or absence of TAD in cytoplasmic domain [1–5]. Notch signaling pathway is activated when the ligands of Delta or Jagged family from neighboring cells bind the receptors and trigger the proteolytic cleavage of Notch receptor. Notch intracellular domain (NICD) is further cleaved by γ -secretase complex as an active moiety and translocated to the nucleus, where it interacts with the DNA binding protein-CBF1 (CSL) via the Notch ankyrin repeats. Interaction of NICD with CBF1 causes displacement of transcriptional co-repressors including silencing mediator of retinoid and thyroid receptors (SMRT) and CIR, whereas the transcriptional co-activators including p300, pCAF and Mastermind like-1 (MAML-1) form active complexes with NICD and CBF1, resulting in the transcription of a small set of downstream target genes. The known targets include Hes1, Hes5 and Hes-related repressor proteins HERP1 and 2. Since CBF-1 binding sites are found in many genes, it is possible that Notch signaling may activate cyclin D1, p21, GFAP, Nodal, c-Myc and some other genes containing CBF-1 binding sites [6–10]. Furthermore, it has been reported recently that NICD may exert its effects directly on specific target genes in a CBF1-independent manner [9, 11].

The activation of Notch signaling pathway leads to different outcomes ranging from control of proliferation to apoptosis, differentiation, maintenance of stemness and cell fate decision [12]. Deregulation of Notch signaling has been implicated in some genetic diseases and tumorigenesis. Studies show that Notch pathway can function as a dominant oncogene in many tumors such as

T-ALLs [13], breast cancer [14], and colon adenocarcinomas [15]. However, Notch has also been shown to act as a tumor suppressor in skin carcinogenesis [16, 17]. Therefore, the function of Notch signaling in a variety of tumors can be either oncogenic or tumor suppressive, depending on the specific cellular context.

The potential role for Notch signaling pathway in brain tumorigenesis has gained attention only recently. In our previous study, overexpression of Notch1 was observed in astrocytomas. Using siRNA targeting Notch1, we observed the remarkable inhibition of astrocytic glioma cell growth both *in vitro* and *in vivo* [18]. However, it has been reported that Notch2 receptor is highly expressed in medulloblastomas, whereas Notch1 expression is scarce or undetectable, and inhibition of Notch2 signaling suppresses growth of medulloblastoma cells, suggesting that Notch1 and Notch2 may have opposite effects on the tumor growth in a single tumor type [19]. The expression of Notch family members in astrocytomas has not been reported. Therefore, in the current study, the expression of Notch family members in both astrocytomas and medulloblastomas was examined by tissue microarray and Western blot analysis.

Materials and Methods

Tissue Microarray and Immunohistochemical Staining

Tissue microarray was prepared by ChaoYing Biotechnology (Shanxi, China). In each tissue microarray slide, there were 60 cases of astrocytoma including 15 cases each for WHO I, II, III, IV, 10 cases of medulloblastoma and 6 cases of normal brain tissue. For immunohistochemical staining, the slides were deparaffinized and put in the 95°C sodium citricum solution 15 min for antigen retrieval, then incubated overnight at 4°C with the primary antibody against each of the Notch family members [Notch1(C-20), Notch2(25–225), Notch3(C-19), Notch4(M-134), Santa Cruz Biotech, USA, 1:100 dilution], then with biotinylated secondary antibody [goat anti-rabbit IgG(ZDR-5118), rabbit anti-goat IgG(ZDR-5114), Zhongshan Goldenbridge Biotech, China, 1:200 dilution] for 1 h at room temperature, followed by incubation with ABC-peroxidase and diaminobenzidine(Zhongshan Goldenbridge Biotech, China). Finally, the slides were counterstained with hematoxylin. The levels of Notch1, 2, 3, 4 expression in each specimen were scored according to the percentage of immunopositive staining cells counted in five randomly selected high power fields: [–]: no expression, [+]: positive cell ratio < 25%, [++]: positive cell ratio between 26–50%, [+++]: positive cell ratio > 50%.

Freshly Resected Tissue Samples and Western Blot Analysis

Thirty-six freshly resected glioma samples were collected in the Department of Neurosurgery at Tianjin Medical University General Hospital during 2007 and classified according to WHO categories (2000). Tissues and clinical information were obtained as a part of Institute Review Board approved study at the University. There were 7 cases of WHO I grade tumors including 7 cases of pilocytic astrocytomas, 7 cases of WHO II grade tumors including 5 cases of protoplasmic and fibrillary astrocytomas and 2 cases of mixed oligodendroastrocytomas, 8 cases of anaplastic astrocytomas (WHO III grade), 10 cases of glioblastomas (WHO IV grade) and 4 cases of medulloblastoma. Four normal brain tissue specimens were obtained from internal decompression of patients with cerebral injury and temporal lobe resection for epilepsy. Each tissue sample was snap frozen in the liquid nitrogen following resection and stored at -70°C . All the tumors and normal brain tissues were diagnosed by two independent neuropathologists.

Transmembrane protein of each forty milligram tissue samples was extracted using a transmembrane protein extract kit (GENMED, Shanghai, China) and the protein concentration was determined by Lowry method. Forty μg of protein lysates from each sample was subjected to SDS-PAGE on 10% SDS-polyacrylamide gels. The separated proteins were transferred to a PVDF membrane and the membrane was incubated with primary antibodies against Notch1(C-20), Notch2(25–225), Notch3(C-19), Notch4(M-134)(Santa Cruz Biotech, USA, 1:500 dilution), followed by HRP-conjugated secondary antibody [goat anti-rabbit IgG(ZDR-5306), rabbit anti-goat IgG(ZDR-5308), Zhongshan Goldenbridge Biotech, China, 1:1000 dilution]. The specific protein was detected using a SuperSignal protein detection kit (Pierce, USA). After washing, the membrane was reprobed with an antibody against beta-actin (sc-47778, Santa Cruz, USA, 1:500 dilution). The band intensity of specific proteins was quantified after normalization with the intensity of beta-actin.

Results

Immunohistochemistry in Tissue Microarray

Immunohistochemistry showed that Notch1, 2, 3, 4 were not expressed or barely detectable in normal brain tissue samples. Notch1 protein was expressed in 48 of 60 (80%) astrocytomas. In all positive cases, Notch1 was detected in the cytoplasm of neoplastic cells. Notch1 displayed varying degrees of intensity in astrocytoma with different grades and the number of positive staining cells increased with the

ascending order of tumor grade. There were 9 of 15 (60%) in WHO I gliomas, 10 of 15 (66.7%) in WHO II, 14 of 15 (93.3%) in WHO III and 15 of 15 (100%) in WHO IV, whereas Notch1 was expressed rarely in medulloblastomas (1 of 10, 10%).

Notch2 protein expression were infrequently detected or expressed in low level in astrocytomas. There were 0 of 15 (0%) in WHO IV, 1 of 15 (6.7%) in WHO III, 2 of 15 (13.3%) in WHO II and 3 of 15 (20%) in WHO I. However, overexpression of Notch2 was detected in the majority of medulloblastomas (9/10, 90%).

Notch3 protein expression could be detected in 47 of 60 (78.3%) astrocytomas. In all positive cases, Notch3 was expressed in the cytoplasm of neoplastic cells. The intensity of expression and the positive staining cell number of Notch3 in astrocytomas was associated with the degree of malignancy of tumors. There were 15 of 15 (100%) in WHO IV, 14 of 15 (93.3%) in WHO III, 10 of 15 (66.7%) in WHO II and 8 of 15 (53.3%) in WHO I. A low level of Notch3 expression was observed in a small fraction of medulloblastomas (2 of 10, 20%).

Notch4 protein expression was detected in 47 of 60 (78.3%) astrocytomas. In all positive cases, Notch4 was expressed in the cytoplasm of tumor cells. Similar to the expression of Notch1 and Notch3 in astrocytomas, Notch4 expression level was also positively correlated with the tumor grade. There were 15 of 15 (100%) in WHO IV, 14 of 15 (93.3%) in WHO III, 11 of 15 (73.3%) in WHO II and 7 of 15 (46.7%) in WHO I. However, Notch4 expression was rarely observed in medulloblastomas (1 of 10, 10%) (Fig. 1; Table 1).

Western Blot Analysis

Notch1, 3, 4 protein were detected in all the astrocytoma tissue samples and their intensity was correlated with increasing grades of glioma malignancy, whereas Notch2 was undetectable. On the contrary, overexpression of Notch2 protein, while no expression of Notch1, 3, 4, was detected in all medulloblastomas. In addition, expression of Notch1, 2, 3, 4 was not detected in normal brain tissues (Fig. 2, Table 2).

Discussion

The prognosis of patients with malignant gliomas remains poor even with the advances in surgical approaches, radiation therapy and chemotherapy. Strategies targeting dysregulated molecular signaling are desperately needed to improve the outcomes of these devastating tumors. In our current study, we showed that Notch family members were differentially expressed in astrocytoma and medulloblasto-

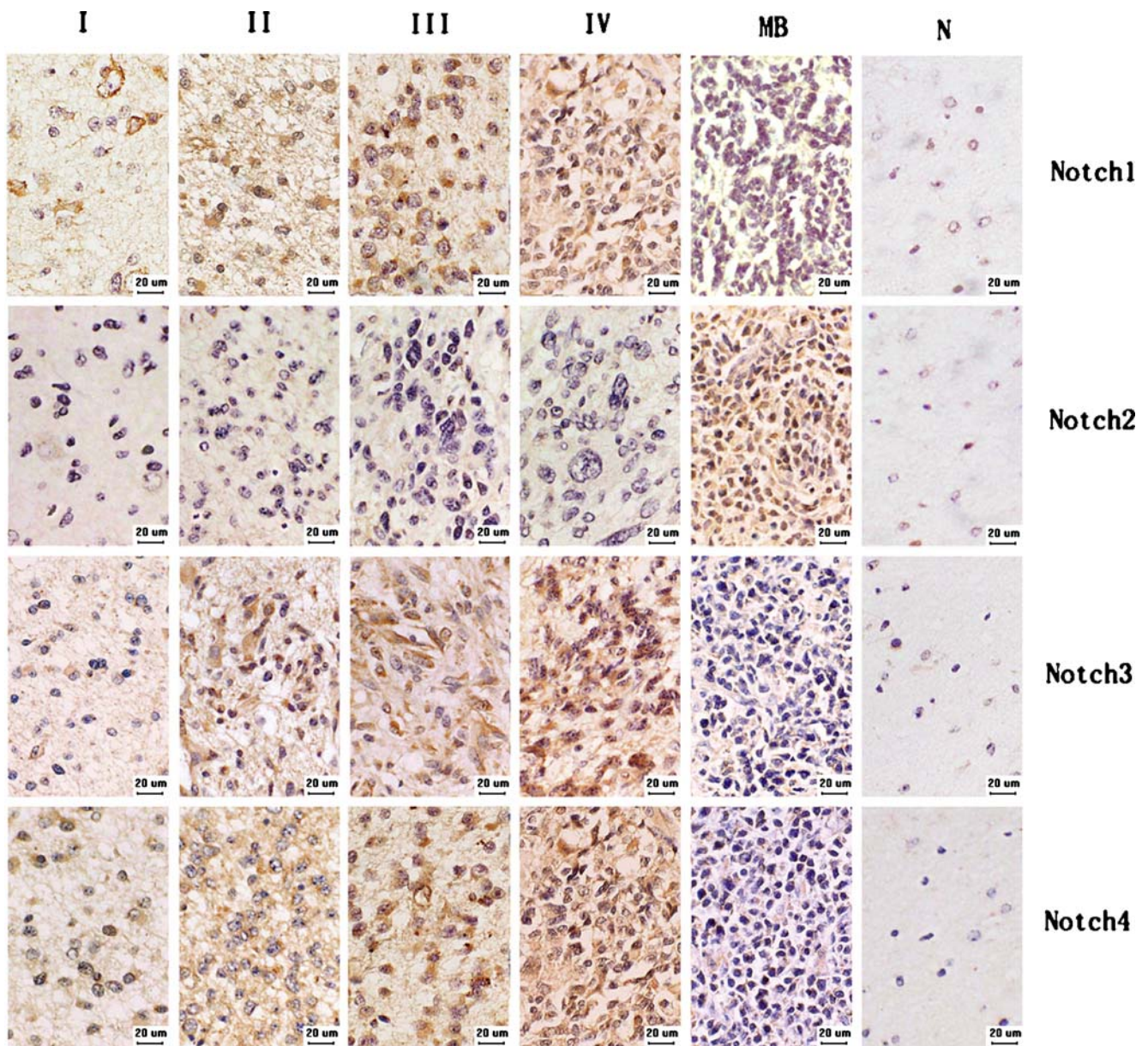


Fig. 1 The expression of Notch1, 2, 3, 4 in the astrocytomas WHO grade I–IV, medulloblastomas and normal brain tissues as detected by immunohistochemical staining ($\times 200$). Notch family members were not expressed or barely detected in normal brain tissues. Notch1, 3, 4 were highly expressed but Notch2 was not expressed in astrocytomas.

ma, but not expressed or barely detectable in normal brain tissues. Notch1, 3, 4 were highly expressed but Notch2 was not expressed in astrocytomas. The percentage of immunopositive tumor cells and level of Notch1 expression was increased with tumor grade. On the other hand, overexpression of Notch2 was detected in medulloblastomas in contrast with low or no expression of Notch1, 3, 4.

Aberrant Notch signaling pathway is found in hematologic malignancies [13] and deregulated Notch receptor expression has been reported in a growing number of

The percentage of positive tumor cells and level of Notch1 expression was increased with tumor grade. On the other hand, overexpression of Notch2 was detected in medulloblastomas in contrast with low or no expression of Notch1, 3, 4

human solid tumors. Increased Notch1 expression and overexpression of Notch2 have been observed in human cervix, colon, lung and pancreatic cancer [15, 20]. Overexpression of Notch3 and Notch4 has been reported in human malignant melanoma [21] and pancreatic cancer [20]. Notch4 mRNA expression has also been found in human breast cancer [22]. Although Notch signaling has mostly been associated with oncogenic and growth-promoting roles, it can also function as a tumor suppressor depending on the tissue type, such as Notch1 in the skin

Table 1 Notch1, 2, 3, 4 expression in astrocytomas WHO grade I-IV, medulloblastomas and normal brain tissue as detected by immunohistochemistry

Target grade		n				Positive rate	total	χ^2 value	P value
		-	+	++	+++				
Notch1	N	6	0	0	0	0	6	10.883	0.013
	AST	I	6	6	3	0	60%	15	
		II	5	7	3	0	66.7%	15	
		III	1	6	7	1	93.3%	15	
		IV	0	4	6	5	100%	15	
M	9	1	0	0	10%	10			
Notch2	N	6	0	0	0	0	6	-	-
	AST	I	12	3	0	0	33.3%	15	
		II	13	2	0	0	20%	15	
		III	14	1	0	0	6.7%	15	
		IV	15	0	0	0	0	15	
MB	1	1	3	5	90%	10			
Notch3	N	6	0	0	0	0	6	12.864	0.005
	AST	I	7	5	3	0	53.3%	15	
		II	5	7	3	0	66.7%	15	
		III	1	7	6	1	93.3%	15	
		IV	0	3	7	5	100%	15	
MB	8	2	0	0	20%	10			
Notch4	N	6	0	0	0	0	6	15.221	0.002
	AST	I	8	5	2	0	46.7%	15	
		II	4	7	4	0	73.3%	15	
		III	1	6	6	2	93.3%	15	
		IV	0	3	6	6	100%	15	
MB	9	1	0	0	10%	10			

N normal brain;AST astrocytomas;MB medulloblastomas

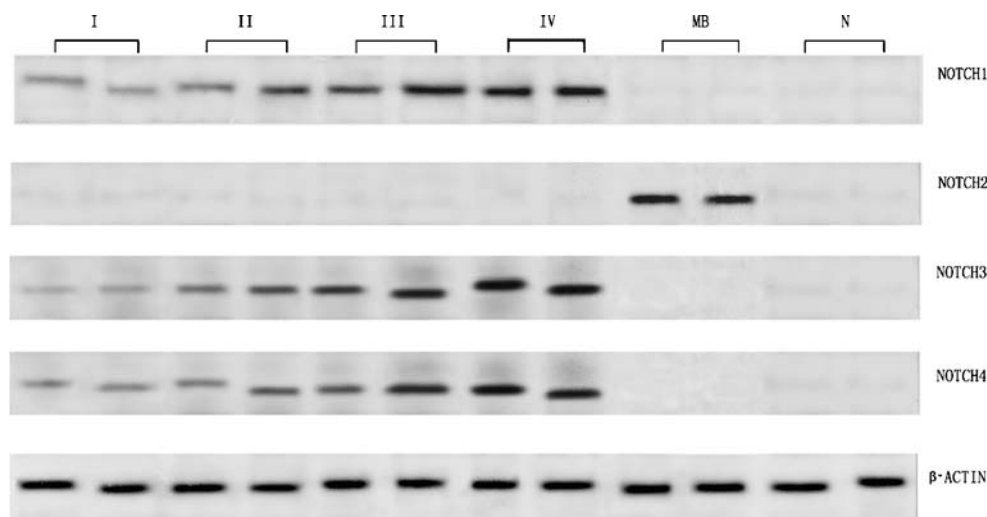
The immunopositive cell number of Notch1, 3, 4 was increased and associated with the degree of malignancy of tumors. The differences of percentage of immunopositive cells and expression level between the low-grade and high-grade tumors were statistically significant by analysis of chi-square test (Notch1: $\chi^2 = 10.883$, $P < 0.05$; Notch3: $\chi^2 = 12.864$, $P < 0.05$; Notch4: $\chi^2 = 15.221$, $P < 0.05$). There is positive correlation between tumor grade and level of Notch1, 3, 4 expression as analyzed by Spearman correlation test. (Notch1: $r = 0.882$, $P < 0.05$; Notch3: $r = 0.882$, $P < 0.05$; Notch4: $r = 0.895$, $P < 0.05$)

carcinogenesis [16] and some neuroendocrine tumors [23]. However, the function of Notch family members in tumorigenesis remains unclear. A major challenge in the study of Notch signaling is to understand how this simple, direct pathway yields such varied outcomes in different cellular context.

A few studies on Notch1 expression in brain tumors have been reported. Induction of members of Notch signaling pathway has been studied in meningiomas using serial analysis of gene expression (SAGE), RT-PCR and quantitative PCR. It is demonstrated that Notch2 and Jagged 1 are the main components expressed in nonneoplastic meninges and meningiomas, while Notch1 homologue is expressed at much lower levels as detected by RT-PCR and quantitative PCR. As examined by Western blot analysis, meninges express higher

levels of Notch2 than Notch1, which is in contrast to those of the normal brain, and both Notch2 and Notch1 are overexpressed in some meningiomas as compared to normal meninges, suggesting that both Notch1 and Notch2 possibly contribute to the meningioma tumorigenesis [24]. Fan et al. reported that Notch1 expression was rarely detected or undetectable, whereas Notch2 was highly expressed in medulloblastoma/PNET tumors. In medulloblastomas, expression studies using primary medulloblastoma tumor samples demonstrated an increase of mRNA expression of Notch2 but not of Notch1. In 15% of the tumors examined, increased Notch2 expression levels were the result of Notch2 gene amplification. Moreover, the proliferation activity of medulloblastoma cells was inhibited by transfection with Notch1 NICD and enhanced by transfection with Notch2

Fig. 2 The expression of Notch1, 2, 3, 4 in the astrocytomas WHO grade I–IV, medulloblastomas and normal brain tissues as detected by Western Blot analysis. There was no expression of Notch family members in normal brain tissues. Notch1, 3, 4 protein were detected in all the astrocytoma tissue samples and their intensity was correlated with increasing grades of glioma malignancy whereas Notch2 was undetectable. On the contrary, overexpression of Notch2 protein was detected in all medulloblastomas, while no expression of Notch1, 3, 4 was observed



NICD *in vitro*. These findings indicate that Notch1 and Notch2 may have opposite effects on the growth of a single tumor type [19]. It has also been reported that Notch1 and its ligands Delta 1 and Jagged 1 were overexpressed in glioma cell lines and primary human gliomas. Knockdown of Notch1, Delta 1 and Jagged 1 by siRNA inhibited

proliferation and induced apoptosis in many glioma cell lines. Implantation of U251MG cells transfected with Notch1, Delta 1 and Jagged 1 siRNA into the brains of nude mice significantly prolonged the survival of animals. These evidences further suggest that Notch1 plays an oncogenic role in the development and progression of

Table 2 Notch1, 2, 3, 4 expression in the astrocytomas WHO grade I–IV, medulloblastomas and normal brain tissue as detected by Western blot analysis

Taget grade	n	Expression n	Expression level (x ± s, %)	F value	P value	
Notch1	N	4	0 (0)	0	146.622	0.000
	AST	I	7	6 (85.7%)	40.72±1.99	
		II	7	7 (100%)	66.85±8.71	
		III	8	8 (100%)	87.31±7.39	
		IV	10	10 (100%)	97.33±3.09	
MB	4	0 (0)	0			
Notch2	N	4	0 (0)	0	-	-
	AST	I	7	0 (0)	0	
		II	7	0 (0)	0	
		III	8	0 (0)	0	
		IV	10	0 (0)	0	
MB	4	4 (100%)	96.97±2.93			
Notch3	N	4	0 (0)	0	152.453	0.000
	AST	I	7	7 (100%)	32.72±2.54	
		II	7	7 (100%)	63.57±6.26	
		III	8	8 (100%)	84.19±5.28	
		IV	10	10 (100%)	96.29±3.19	
MB	4	0 (0)	0			
Notch4	N	4	0 (0)	0	173.342	0.000
	AST	I	7	6 (85.7%)	36.36±4.37	
		II	7	7 (100%)	65.32±5.28	
		III	8	8 (100%)	86.37±6.26	
		IV	10	10 (100%)	97.15±4.52	
MB	4	0 (0)	0			

N normal brain;AST astrocytomas;MB medulloblastomas

The differences of expression level of Notch family members in low-grade and high-grade tumors were statistically significant by analysis of variance. (Notch1:F=146.662, P<0.05; Notch3:F=152.453, P<0.05; Notch4:F=173.342, P<0.05)

There is a positive correlation between tumor grade and expression of Notch1, 3, 4 as analyzed by Spearman correlation test

(Notch1: r=0.930, P<0.05; Notch3: r=0.930, P<0.05; Notch4: r=0.945, P<0.05)

astrocytic gliomas [25]. However, There have been reported that a few glioblastoma samples do not express Notch1 or express both Notch1 and Notch2 [26, 27]. Dang et al. demonstrated that introduction of constitutively active Notch3 into periventricular cells of embryonic day 9.5 mice caused the formation of choroid plexus tumors, suggesting that Notch3 activity might present in human choroid plexus lesions [28]. There is no report about the function of Notch4 in brain tumorigenesis. Therefore, differentially expressed Notch family members may play diverse roles in various types of brain tumors.

In our present study, Notch1, 2, 3, 4 were not expressed in normal brain tissue samples. Notch1, 3, 4 were highly expressed but Notch2 was not expressed in astrocytomas. The percentage of positive tumor cells and intensity of Notch1 expression were increased with tumor grade. In addition, overexpression of Notch2 was detected in medulloblastoma in contrast with low or no expression of Notch1, 3, 4, indicating that there were differential expression of Notch family members in astrocytomas and medulloblastomas. It is speculated that these results may be related to their different functional activities during the brain development. In this scenario, Notch could act as an oncogene if the cells are derived from a tissue in which Notch normally functions as a gatekeeper of stem cells, or influences cell-fate decision at a precursor level. The tumor-suppressor activity, on the other hand, would be manifested in tissues in which Notch signaling initiates terminal differentiation events [29]. In the development of mice brain, activated Notch1 regulates the magnitude of neurogenesis from postnatal progenitor cells. Abrogation of Notch signaling leads to a transition from neural stem or precursor cells to transit-amplifying cells or neurons [30]. On the other hand, activated Notch1 is mainly detected in the nuclei of a subpopulation of radial glial cells and necessary for gliogenesis because it could promote the expression of a glial-specification gene [31, 32]. In the developing cerebellar cortex, granule neuron precursors (GNPs) proliferate and commence differentiation in a superficial zone, the external granule layer (EGL). Notch2 is specifically expressed in proliferating GNPs which is considered as the original site of the medulloblastoma coming from the EGL [33, 34]. Notch 3 has a more restricted distribution in vascular smooth muscles, central nervous system and regulatory T cells etc. Its activating genetic mutations results in human pathologies similar to other members of the family. However, the molecular mechanisms remain elusive [35]. Up to date, the function of Notch family members in various types of brain tumors is still largely undefined and needs investigate further.

Recent studies have promoted major advances for our understanding of dual function of Notch signaling in oncogenesis, its cross-talk and complex linking with other

signaling pathways [36, 37]. It is expected that more detailed studies on Notch signaling will benefit the development of new therapeutic approaches for gliomas.

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