LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

The oncogenic roles of Notch1 in astrocytic gliomas in vitro and in vivo

**Peng Xu · Mingzhe Qiu · Zhiyong Zhang ·
Chunsheng Kang · Rongcai Jiang · Zhifan Jia ·
Guangxiu Wang · Hao Jiang · Peiyu Pu**

Abstract Notch receptors play an essential role in cellular processes during embryonic and postnatal development, including maintenance of stem cell self-renewal, proliferation, and determination of cell fate and apoptosis. Deregulation of Notch signaling has been implicated in some genetic diseases and tumorigenesis. The function of Notch signaling in a variety of tumors can be either oncogenic or tumor-suppressive, depending on the cellular context. In this study, Notch1 overexpression was observed in the majority of 45 astrocytic gliomas with different grades and in U251MG glioma cells. Transfection of siRNA targeting Notch1 into U251 cells in vitro downregulated Notch1 expression, associated with inhibition of cell growth, arrest of cell cycle, reduction of cell invasiveness, and induction of cell apoptosis. Meanwhile, tumor growth was delayed in established subcutaneous gliomas in nude mice treated with Notch1 siRNA in vivo. These results suggest that Notch1 plays an important oncogenic role in the development and progression of astrocytic gliomas. Furthermore, knockdown of Notch1 expression by siRNA simultaneously downregulated the expression of EGFR and the important components of its downstream pathways, including PI3K, p-AKT, K-Ras, cyclin D1 and MMP9, indicating the crosstalk and interaction of Notch and EGFR signaling pathways.

Keywords Glioma · Notch1 · siRNA · EGFR

Peng Xu and Mingzhe Qiu contributed equally to this work.

P. Xu · M. Qiu · Z. Zhang · C. Kang · R. Jiang ·
Z. Jia · G. Wang · P. Pu (✉)
Department of Neurosurgery, Tianjin Medical University
General Hospital, 154 Anshan Road, Tianjin 300052,
People's Republic of China
e-mail: pupeiyu33@hotmail.com; pupeiyu@gmail.com

P. Xu · M. Qiu · Z. Zhang · C. Kang · R. Jiang ·
Z. Jia · G. Wang · P. Pu
Laboratory of Neuro-Oncology, Tianjin Neurological Institute,
Tianjin 300052, People's Republic of China

H. Jiang
Department of Neurology, Henry Ford Hospital,
Detroit, MI 48202, USA

Notch proteins are cell-surface receptors that play essential roles in cellular processes during embryonic and postnatal development, maintain the stem cells in the undifferentiated state, participate in binary cell-fate decision, and induce terminal differentiation. In mammals, there are four highly homologous Notch receptors, Notch1 to 4, and five ligands, Delta-like 1, 3, and 4, and Jagged 1 and 2 [1–5]. The Notch signaling pathway is activated when a ligand of the Delta or Jagged family from neighboring cells binds the receptors and leads to successive proteolytic changes. Finally, the Notch intracellular domain (NICD) is generated by γ-secretase cleavage to form an active moiety and translocate to the nucleus, where it interacts with the DNA binding protein-CBF1 via the Notch ankyrin repeats. Binding of NICD with CBF1 causes displacement of transcriptional co-repressors, including SMRT (silencing mediator of retinoid and thyroid receptors) and CIR (CBF1 interacting corepressor). The transcriptional co-activators, including p300, pCAF, and Mastermind-1 (MAML-1), form active complexes with NICD and CBF1, resulting in the transcription of a small set of downstream target genes, for example Hes1 and Hes5, and Hes-related repressor proteins HERP1 and 2. Because CBF-1 binding sites are found in many genes, it is possible that Notch signaling may activate cyclin D1, p21, GFAP, Nodal, and some other genes [4, 6–8]. Furthermore, it has been reported recently

that NCID may exert its effect directly on specific target proteins in a CBF1-independent manner [5, 8].

Accumulating evidence has shown that Notch signaling plays an important role in a wide range of human neoplasms including brain tumors [4, 9–20]. In our previous study, the gene-expression profiles of 63 samples of different pathological types of human neuroepithelial tumors and five samples of human normal brain tissues were selectively analyzed by Atlas Human Cancer Array 1.2 [21]. It had been observed that some members of Notch pathway were differentially expressed in gliomas. In this study, we further evaluate the role of Notch signaling in gliomagenesis by analyzing the expression of Notch1 in more samples of primary astrocytic gliomas. We found that Notch1 was significantly overexpressed in gliomas. In addition, the effects of downregulation of Notch1 expression by siRNA in cultured malignant glioma cells and established subcutaneous gliomas in nude mice were studied.

Materials and methods

Tissue samples

Forty-five freshly resected astrocytic glioma samples were collected in the Department of Neurosurgery at Tianjin Medical University General Hospital during 2004 and classified according to the WHO categories (2000). There were 19 cases of WHO II grade tumors, including 17 cases of protoplasmic and fibrillary astrocytomas and two cases of mixed oligodendroastrocytomas, 13 cases of anaplastic astrocytomas (WHO III grade), and 13 cases of glioblastomas (WHO IV grade). Four normal brain tissue specimens were taken from internal decompression of patients with cerebral injury and temporal lobe epilepsy and verified by histopathological examination as normal controls. A portion of each tissue sample was snap frozen in liquid nitrogen after resection and stored at −70°C for isolation of RNA. The remaining portion was fixed with 10% formalin for histopathological and immunohistochemical examination.

RT-PCR analysis, western blot analysis, and immunohistochemical staining

The expression of Notch1 and correlation with degrees of malignancy in 45 gliomas and four normal brain samples were determined by RT-PCR and immunohistochemical staining.

For RT-PCR, total RNA was extracted from the tissue samples using Trizol reagent (Invitrogen, USA), reverse transcribed to cDNA and amplified by PCR. The PCR amplification conditions for Notch1 were: initial denaturation at 94°C for 5 min, then 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s for 30 cycles; after the final cycle, the reaction was held in 72°C for 5 min. A portion of β-actin gene was also amplified under the same conditions as a control. PCR primer set used to amplify each gene was as follows: Notch1: 5′-CCGCAAGCCCAGCAGCAAA-3′ and 5′-GGACCCGCCCACAGTGAAAT-3′; β-actin: 5′-GCCG GGACCTGACTGACTA-3′ and 5′-TGCGGATGTCCACG TCACACT-3′. The resulting PCR products were electrophoresed on a 2% agarose gel, which was stained with ethidium bromide, digitally photographed, and scanned with UVi Gel analyzing system (UK). The level of gene expression was calculated as the ratio of mean band density of specific RT-PCR products to that of the internal β-actin standard.

Transmembrane protein of each 40-mg tissue sample was extracted according to the manual of the transmembrane protein extract kit (GENMED, Shanghai, China) and protein concentration was determined by the Lowry method. Forty microgram of protein lysates from each sample was subjected to SDS-PAGE on 10% SDS–polyacrylamide gel. The separated proteins were transferred to a PVDF membrane and the membrane was incubated with primary antibodies against Notch1 (Santa Cruz Biotechnology, USA, 1:500 dilution), followed by HRP-conjugated secondary protein (Zymed, USA, 1:1000 dilution). The specific protein was detected using a SuperSignal protein detection kit (Pierce). After washing, the membrane was rehybridized with a primary antibody against β-actin (Santa Cruz Biotechnology, USA; 1:500 dilution), using the same procedures as described above. The band density of specific proteins was quantified after normalization with the density of β-actin.

Formalin fixed tissue samples were prepared as paraffin-embedded sections and stained with hematoxylin and eosin for histopathological diagnosis. Unstained sections were deparaffinized and subjected to immunohistochemical staining. The sections were incubated overnight at 4°C with Notch1 primary antibody (Santa Cruz Biotechnology; 1:100 dilution) and then at room temperature for 1 h with biotinylated secondary antibody (1:200 dilution), followed by incubation with ABC-peroxidase and diaminobenzedine. Sections were counterstained with hematoxylin. Notch1 expression in each specimen was scored according to the percentage of the positive staining cells counted in five randomly selected high magnificent fields: 0—no expression, 1—positive cell ratio <25%, 2—positive cell ratio between 26 and 50%, 3—positive cell ratio >50%.

Cell culture and transfection

Human glioblastoma U251-MG cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were cultured in DMEM

supplemented with 10% fetal calf serum. Twenty-four hours after plating in six-well plates, cells were transfected with 20 ng/10 μl siRNA in serum-free medium mediated by lipofectamin (Invitrogen, USA). Six hours later, serum-free medium was replaced with DMEM supplemented with 10% fetal calf serum and cells were incubated at 37°C in 5% CO_2. The siRNA sequence targeting Notch1 was 5′-UGGCGGGAAGUGUGAAGCG-3′ (Gima Biol Engineering, Shanghai, China) and a scramble siRNA sequence (5′-TTCTCCGAACGTGTCACGT-3′) was used as a control.

Western blot analysis

Total proteins were extracted from U251 glioma cells, and from U251 cells transfected with scramble siRNA and Notch1 siRNA. Notch1, EGFR, PI3K, phosphorylated AKT (p-AKT), K-Ras, and cyclin D1 (all antibodies from Santa Cruz Biotechnology) were detected according the methods described above.

Immunofluorescence staining

Forty-eight to seventy-two hours after transfection, parental cells and scramble or Notch1 siRNA-transfected cells grown on the coverslips were fixed with methanol at −20°C, blocked with 1% BSA-PBS for 30 min, incubated with the primary antibodies (1:100 dilution) against Notch1, EGFR, PI3K, p-AKT, K-Ras, and cyclin D1 at 4°C overnight, incubated at 37°C for 30 min with TIRTC (tetramethylrhodamine isothiocyanate)-labeled secondary antibody at 1:200 dilution, washed with PBS, then mounted with 0.5 M Na_2CO_3–50% glycerol and examined by fluorescence microscopy.

MTT [3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide] assay

The growth rate of control and siRNA-transfected U251 cells was measured by MTT assay. Briefly, 4×10^3 cells per well were plated into a 96-well plate. At 24, 48, and 72 h after plating, 20 μl (5 mg MTT/ml) was added to each well and the cells were incubated at 37°C for an additional 4 h. The reaction was then terminated by lysing the cells with 200 μl DMSO for 5 min. Optical density was measured in triplicate at 570 nm and expressed as a percentage of the control value.

Flow-cytometric analysis of cell-cycle kinetics

Parental and transfected U251 cells in the log phase of growth were harvested and incubated with RNase at 37°C for 30 min. The cell nuclei were stained with propidium iodide for an additional 30 min. A total of 10,000 nuclei were analyzed by use of a FacsCalibur flow cytometer and the DNA histograms were generated by Modifit software (Becton-Dickinson, USA).

Measurement of apoptosis by annexin V and TUNEL staining

Annexin V-cy3-labeled Apoptosis Detection Kit 1 (Abcam, USA) was used for detection of apoptotic cells by flow cytometry as described previously [22]. Data were analyzed by use of Cell Quest software (Becton-Dickinson, USA). The extent of apoptosis in the tumor specimens of mouse models from the in-vivo study was evaluated by the TUNEL method using an in situ Cell Death Kit (Roche, USA). Cell nuclei were counterstained with Hoechst 33342, visualized by fluorescent microscopy, and analyzed by IPP5.1 (Olympus, Japan).

Three dimensional cell growth on Matrigel

Each well of a 24-well plate was precoated with 200 μl undiluted phenol-red free Matrigel (BD Biosciences, USA). Six hours after transfection with siRNA, cells were harvested, diluted to a concentration of 2×10^4 per well in 200 μl complete medium, mixed with 100 μl undiluted ice-cold Matrigel in the ratio 2:1 (*v/v*), and laid over the bottom layer. After gelling, complete culture medium was added and changed every 2–3 days. Cell morphology was assessed on day 14 by images captured at ×40 magnification.

Establishment of subcutaneous glioma model and treatment with siRNA targeting Notch1

Six-week-old female immune-deficient nude mice (BALB/C-nu) were purchased from the animal center of the Cancer Institute, Chinese Academy of Sciences, bred at the facility of laboratory animals, Tianjin Medical University, and housed in microisolator individually ventilated cages with water and food. All experiments were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and Tianjin Municipal Science and Technology Commission.

A subcutaneous U251 glioma xenograft model was established as described previously [22]. When the tumor size reached approximately 5 mm in diameter, the mice were randomly divided into three groups:

1. control group with tumors untreated;
2. Notch1 siRNA-treated group—mice were injected intratumorally with 25 μl siRNA/oligofectamine mixture containing 400 pmol siRNA every 4 days; and
3. scramble siRNA (scr-siRNA)-treated group. The same dosage of scramble siRNA was used as that in Notch1 siRNA-treated group.

Each group consisted of eight mice. During 32 days of observation the tumor volume was measured with a caliper every three days using the formula: volume = length × width^2/2.

At the end of observation period, the mice were killed and the removed tumor specimens were prepared as paraffin-embedded sections for detection of the expression of Notch1, EGFR, PI3K, p-AKT, K-Ras, MMP9, cyclin D1, PCNA, and GFAP by immunohistochemical staining. Apoptosis in the tumor specimens was determined by the TUNEL method as previously described.

Results

Expression of Notch1, PCNA, and cyclin D1 in gliomas

As shown by RT-PCR, western blot, and immunohistochemical staining, in normal brain tissues, Notch1 expression was not detected or was only faintly detected at mRNA and protein levels. However, Notch1 expression was significantly increased in gliomas, and its expression was higher in high-grade astrocytomas than that in low-grade ones. Meanwhile, Notch1 expression in gliomas was positively correlated with the proliferation activity of tumors as indicated by PCNA immunostaining and the expression of its downstream target, cyclin D1 (Fig. 1a, b, c; Tables 1, 2).

Table 1 Notch1, cyclin D1, and PCNA expression detected by immunohistochemistry in 45 astrocytic gliomas with different grades of malignancy

	Grade	Cases	Positive rate (%)	Positive constitute ratio			
				−	+	++	+++
Notch1	II	19	68.4	6	8	5	0
	III	13	84.6	2	4	5	2
	IV	13	92.3	1	3	5	4
Cyclin D1	II	19	63.2	7	9	6	0
	III	13	76.9	3	4	5	1
	IV	13	92.3	1	4	4	4
PCNA	II	19	73.7	5	9	5	0
	III	13	84.6	2	5	5	1
	IV	13	92.3	1	2	5	5

Notch1: $\chi^2 = 14.213$, $P < 0.05$; Cyclin D1: $\chi^2 = 17..521$, $P < 0.05$; PCNA: $\chi^2 = 16.184$, $P < 0.05$

Table 2 Notch1 expression detected by western blot analysis in 45 astrocytic gliomas with different grades of malignancy

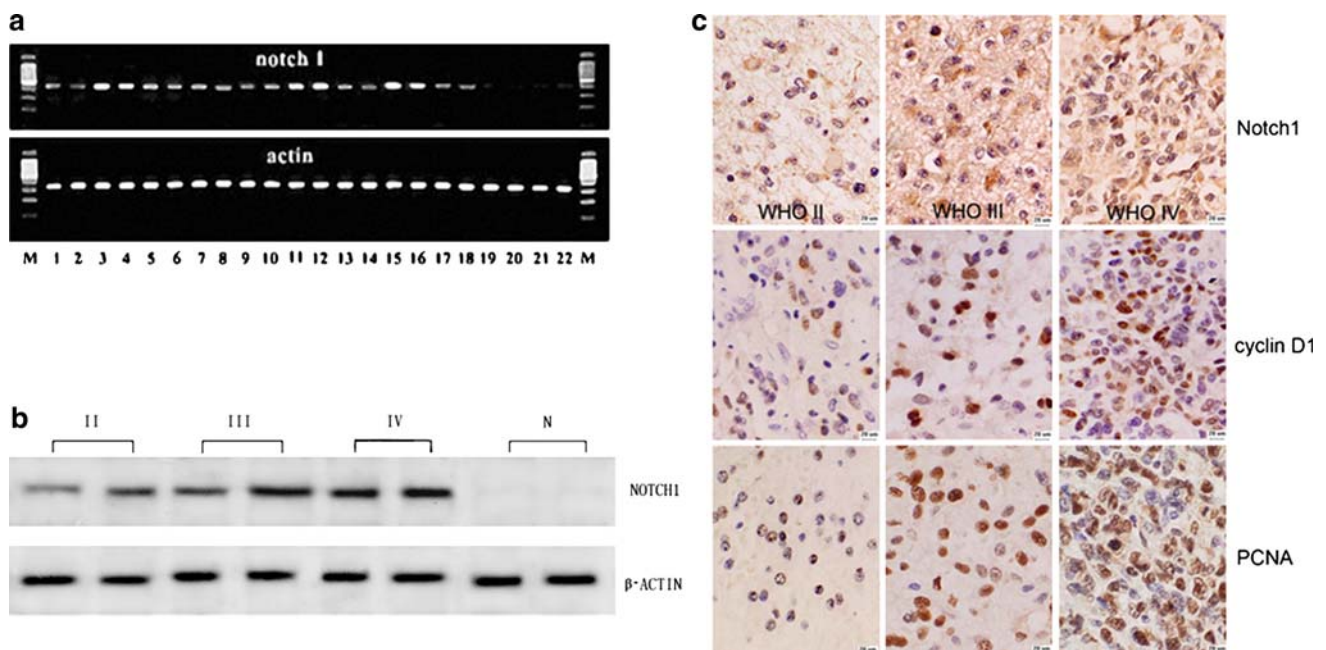| Grade	Cases	Fold ($x \pm s$)	F	P
II	19	0.30 ± 0.09		
III	13	0.58 ± 0.05	12.640	<0.001
IV	13	0.72 ± 0.07		

Fig. 1 Notch1 expression by RT-PCR, western blot, and immunohistochemistry staining in astrocytic gliomas. **a** Notch1 mRNA expression by different grades of astrocytic gliomas examined by RT-PCR. *M* marker, *1–6* WHO IV gliomas, *7–12* WHO III gliomas, *13–18* WHO II gliomas, *19–22* normal brain tissues. **b** The expression of Notch1 by astrocytoma grade II–IV and normal brain tissues as demonstrated by western blot analysis. **c** Notch1, cyclin D1, and PCNA expression by different grades of astrocytic gliomas detected by immunohistochemistry (×200)

Expression of Notch1 and related proteins in control and siRNA transfected U251 cells

After transfection with Notch1 siRNA, expression of Notch1 and its direct effector, cyclin D1, and expression of EGFR and the major members of its downstream pathways including PI3K/p-AKT and K-Ras was markedly reduced to a lower level in U251 cells, detected with either western blotting or immunofluorescence staining, as shown in Fig. 2a, b.

Effect of Notch1 knockdown on U251 cell proliferation, apoptosis, and invasion

The proliferation activity of U251 cells was examined by MTT assay after downregulation of Notch1 expression by transfection of Notch1 siRNA. As shown in Fig. 3a, the growth rate of cells transfected with scramble siRNA was not affected. However, the proliferation of cells transfected with Notch1 siRNA was inhibited at 24 h after transfection and tended to be more significant at 48 and 72 h after transfection.

Cell cycle kinetics examined by flow cytometry showed that downregulation of Notch1 expression of U251 cells by siRNA resulted in the decrease of the S phase fraction and arrest of cells in the G0/G1 phase (Fig. 3b), whereas the number of apoptotic cells evaluated by annexin V labeling was significantly increased (Fig. 3c).

The invasive ability of parental and transfected cells was assessed by in-vitro 3D growth in Matrigel matrix. U251 cells and cells transfected with scramble siRNA grew as aggregated colonies from which highly invasive cells emanated to form stellate like extensions, while the cells transfected by Notch1 siRNA formed colonies without or with only limited outgrowth of invasive cells. This observation suggests that knockdown of Notch1 expression also attenuates the aggressive capability of malignant glioma cells (Fig. 3d).
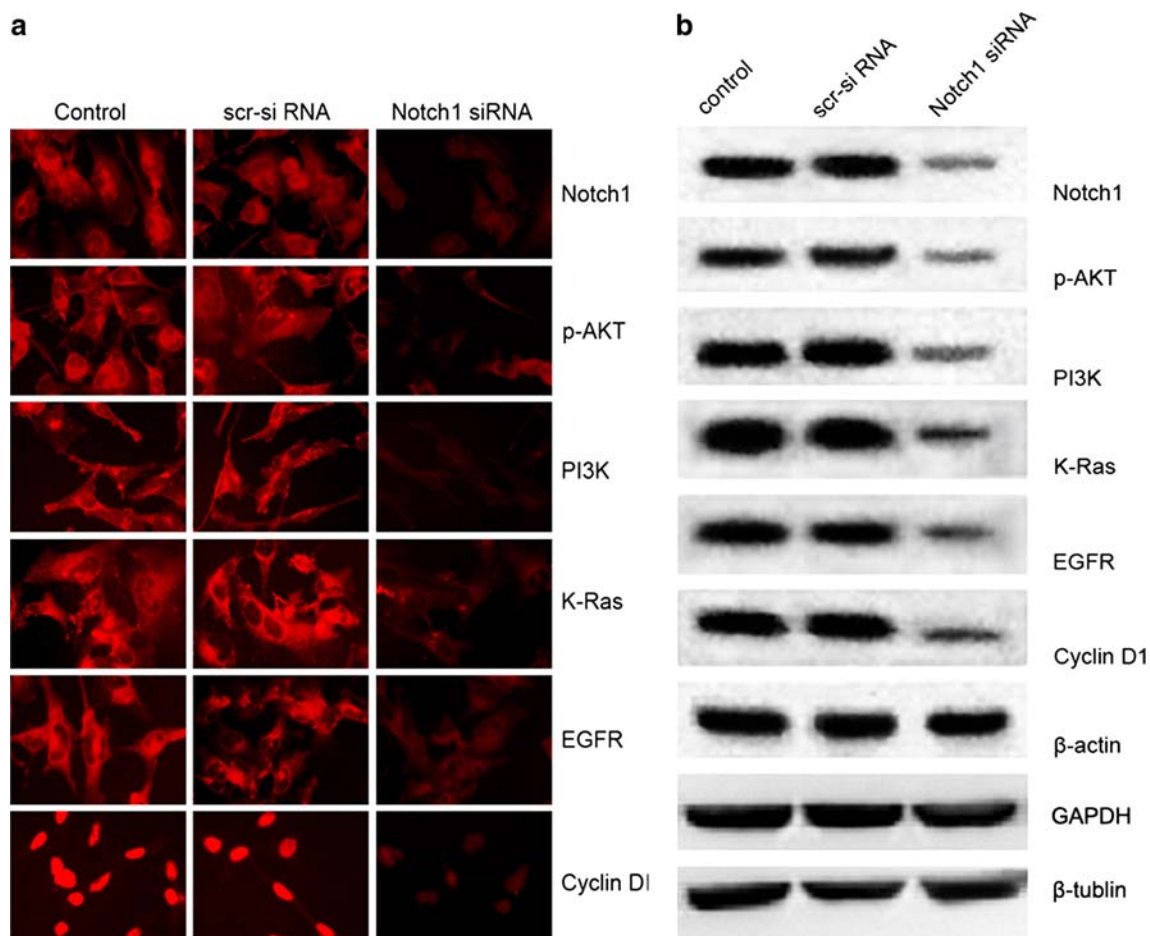

Fig. 2 Expression of Notch1 and relevant genes in U251 cells transfected with Notch1 siRNA by immunofluorescence staining and western blotting. a Notch1, p-AKT, PI3K, K-Ras, EGFR, and cyclin D1 expression of U251 cells transfected with Notch1 siRNA as shown by immunofluorescence staining (×200). b Western blot analysis of Notch1, p-AKT, PI3K, K-Ras, EGFR, and cyclin D1 expression in U251 cells transfected with Notch1 siRNA
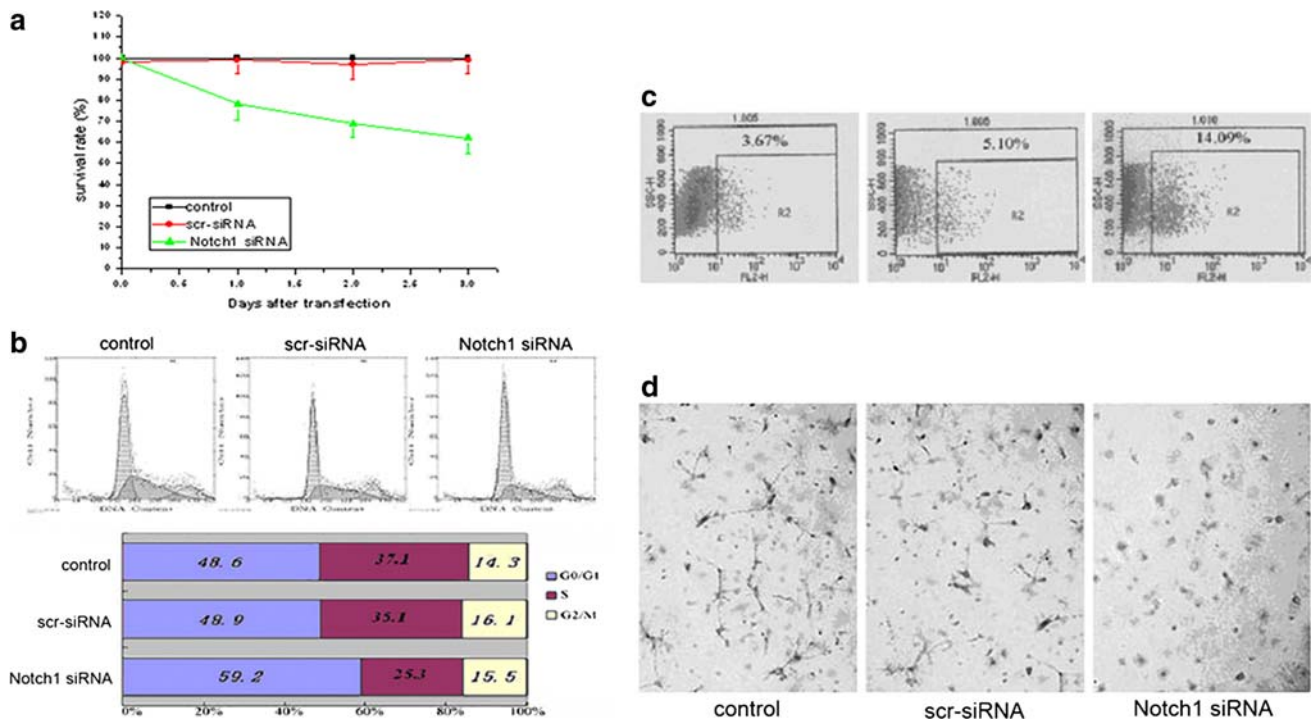
Fig. 3 In-vitro study of proliferation, apoptosis, and invasive ability in U251 cells transfected with Notch1 siRNA. **a** Proliferation rate of U251 cells transfected with Notch1 siRNA determined by MTT assay. **b** Cell cycle analysis of U251 transfected with siRNA targeting Notch1 examined with flow cytometry. **c** Apoptosis of U251 cells transfected with siRNA targeting Notch1 detected with annexin V staining by flow cytometry. **d** Invasive ability of U251 cells and U251 cells transfected with scr-siRNA and Notch1 siRNA examined by three-dimensional cell growth in Matrigel. Control cells and scr-siRNA transfected cells showed penetrating stellate morphology, whereas Notch1 siRNA-transfected cells formed very limited projections

Anti-glioma effect of Notch1 siRNA in vivo

The anti-glioma effect of Notch1 siRNA in vivo was investigated using a U251 subcutaneous glioma xenograft model. Nude mice bearing the largest and smallest tumors were eliminated from the study. The mean volume of the tumors was 81.55 ± 22.86 mm^3 before treatment. During the first two weeks of the observation period, the mice with either control or treated tumors grew slowly and showed no difference in tumor size. As shown in Fig. 4a, at day 16 after implantation, especially from day 24, the tumors in the control and scr-siRNA treated mice had been growing rapidly until to the end of observation period on day 32. One of the mice bearing the largest volume of glioma in the control group died of cachexia. However, the tumors treated with Notch1 siRNA still maintained the slower growth rate and there was a significant difference of tumor volume between the control and Notch1 siRNA-treated mice in the last half of the observation period ($P < 0.01$). The tumors removed from the control and scr-siRNA treated mice exhibited hemorrhage, liquidation, and necrosis macroscopically, whereas the tumors taken from the Notch1-treated mice were solid, and small necrotic foci were rarely seen.

Expression of Notch1 and its related proteins in tumors treated with Notch1 siRNA

Similar to the results obtained from in-vitro studies, the expression of Notch1 and its downstream effector cyclin D1, EGFR and its downstream targets PI3K, p-AKT, and K-Ras, and protease MMP9 in tumor specimens from Notch1 siRNA-treated mice was reduced. In addition, expression of PCNA, a marker of cell proliferation activity, was reduced, whereas GFAP expression was significantly increased (Fig. 4b). These results further demonstrate that crosstalk occurs between Notch and EGFR signaling and the cells move toward differentiation as the GFAP expression level elevated.

Detection of apoptosis in tumor samples treated with Notch1 siRNA

Apoptosis in tumor samples obtained from control and treated mice were examined by TUNEL staining. The number of apoptotic cells in tumors treated with Notch1 siRNA was significantly larger than that in control and scr-siRNA treated mice (Fig. 4c; Table 3).
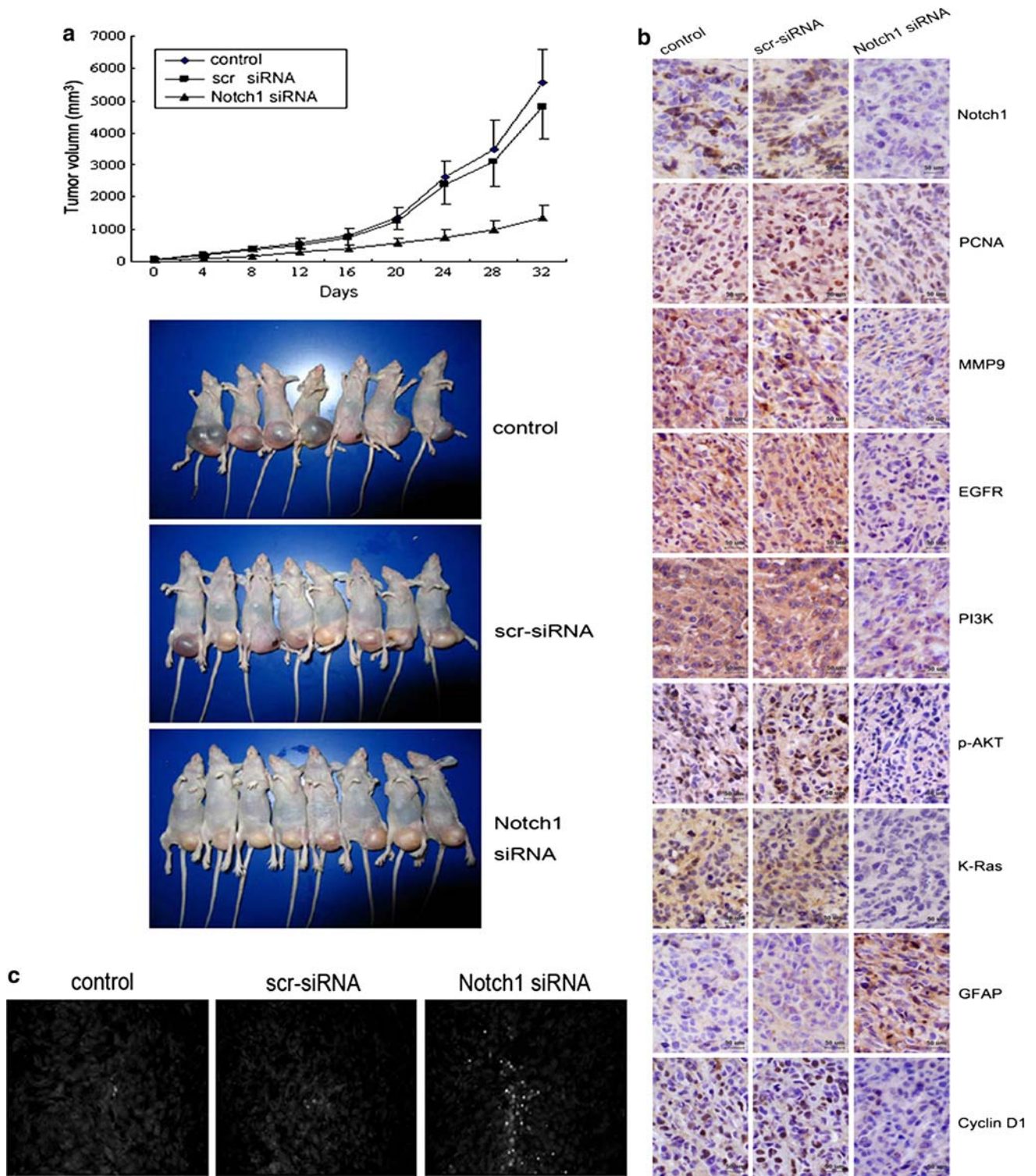
Fig. 4 In-vivo study of nude mice bearing subcutaneous xenograft gliomas treated with Notch1 siRNA. **a** Tumor growth in nude mice treated with Notch1 siRNA compared with that in control and scr-siRNA treated mice. **b** Expression of Notch1, PCNA, MMP9, EGFR, PI3K, p-AKT, K-Ras, GFAP, and Cyclin D1 in Notch1 siRNA-treated tumors compared with that in control and scr-siRNA-treated tumors (×200). **c** Apoptosis cells in xenograft tumors, and in tumors treated with scr-siRNA and Notch1 siRNA, detected by the TUNEL method (×200)

Table 3 Apoptosis index (AI) of U251 cells in xenograft tumors and tumors treated with scrambled siRNA and Notch1 siRNA

Group	n	AI ($x \pm s$)	F	P
Control	7	2.6 ± 1.4		
Scr-siRNA	8	3.9 ± 2.4	79.453	<0.001
Notch1 siRNA	8	$22.1 \pm 3.2*$		

* Compared with control $P < 0.01$

Discussion

Gliomas are the most common primary brain tumors with high morbidity and mortality. Despite advances in the diagnosis and treatment of gliomas during the past several decades, the prognosis of patients remains poor. It is critical to understand the molecular mechanism of gliomagenesis for possible development of novel and promising therapeutic approaches. In our previous study, we observed, by analysis of global gene expression profiles of 63 neuroepithelial tumor specimens with cDNA microarray, that Notch1 and 4 are overexpressed in most gliomas [21]. In addition, it is well known that EGFR aberration is the most frequent and important genetic alteration in gliomagenesis. We have demonstrated that transfection of plasmid-based siRNA targeting the intracellular domain of EGFR (516–536 and 2,400–2,420 bp) into established glioblastoma cell line TJ905 not only knocks down the EGFR expression, but also results in a decrease in Notch1 expression [23], indicating that there is a functional link between EGFR and Notch signaling. To further explore the role of Notch1 in the gliomagenesis and investigate the interactions between EGFR and Notch1, in this study, we examined Notch1 expression in gliomas and the effects of downregulation of Notch1 by siRNA on glioma cell proliferation, invasion, apoptosis, and the EGFR signaling pathway.

Notch signaling is involved in a variety of tumors and functions as a dominant oncogene in some neoplasms, whereas it behaves as a tumor suppressor gene in others [24–26]. For example, constitutive overexpression of active Notch1 in human hepatocellular carcinoma inhibits the proliferation of HCC cells through the arrest of cell cycle, downregulation of cyclin A, D, E, CDK2, and pRb, and upregulation of p21, p53, and Jun-NH2-terminal kinase activation [13]. In contrast, overexpressing Notch1 in tumor cells and binding with its ligand Jagged-1 promotes cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma [16], and Notch1 signaling activation is required for the development of chemically induced pancreatic cancer [27]. It has also been shown that Notch signals are oncogenic in pre-T cells [4, 10] but suppress tumor development in keratinocytes [28, 29]. Therefore, the function of Notch signaling in tumorigenesis can be either oncogenic or tumor-suppressive, largely depending on cellular context [4, 17, 30].

As shown in this study, Notch1 is overexpressed in astrocytic gliomas. In particular, increased expression of Notch1 is correlated with increasing grades of glioma malignancy and the expression of biomarker of proliferation activity, PCNA, and the positive cell cycle regulator, cyclin D1, suggesting that overexpression of Notch1 promotes cell proliferation and acts as an oncogene in astrocytic gliomas. A few studies on Notch1 expression in brain tumors have been reported [18–20]. It is found that both Notch1 mRNA and protein expression are higher in different primary human gliomas than non-neoplastic brains [18]. Induction of members of the Notch signaling pathway has also been studied in meningiomas using serial analysis of gene expression (SAGE), RT-PCR, and quantitative PCR. It has been reported that Notch2 and Jagged 1 are the main components expressed in nonneoplastic meninges and meningiomas, whereas the Notch1 homolog is expressed at much lower levels [19]. These findings indicate that the Notch pathway is deregulated in meningiomas, although the results are somewhat different from those found in astrocytic gliomas. Moreover, the results obtained from astrocytic tumors in this study are also different from those observed in the progenitor cell-derived brain tumors, such as PNET and medulloblastoma. Fan et al. has reported that Notch1 expression is rarely detected, or is undetectable, whereas Notch2 is highly expressed in those tumors [20]. Whether the expression levels and effects of Notch1 in different types of brain tumor are due to the different cellular context and the underlying mechanism are still not clear.

The contribution of Notch1 to gliomagenesis is further demonstrated by downregulation of its expression by small interference RNA (siRNA) in vitro and in vivo. Downregulation of Notch1 expression by siRNA is associated with inhibition of cell proliferation, arrest of cell cycle, reduction of invasiveness of tumor cells, and induction of cell apoptosis, as shown by the in-vitro study. Furthermore, the tumor growth in vivo is delayed after treatment with Notch1 siRNA in established subcutaneous gliomas of nude mice. These results are somewhat similar to those of the study by Purow et al. [18], in which they observed that knockdown of Notch1, Delta 1, and Jagged 1 by siRNA inhibits proliferation and induces apoptosis in glioma cell lines, and implantation of U251MG cells pretreated with Notch1, Delta 1, and Jagged 1 siRNA into the brains of nude mice significantly prolongs survival of the animals. The siRNA sequences targeting Notch1 we used were also adopted from this study and no off-target effect was found. All this evidence further suggests that Notch1 plays an oncogenic role in the development and progression of astrocytic gliomas. However, as above mentioned, Fan

et al. found not only differential expression of Notch1 and Notch2 in medulloblastoma/PNET tumors, but also different Notch receptors, with opposite effects, in a single embryonic brain tumor type. They demonstrated that transfection with constitutively active forms of Notch1 or Notch2 (the intracellular domain of Notch1 and Notch2, NICD1 and NICD2) has antagonistic effects on cell growth in medulloblastoma cell line DAOY. Overexpression of Notch2 promotes cell proliferation, soft agar colony formation, and xenograft growth, whereas these are inhibited by overexpression of Notch1. These findings have been further confirmed by knocking down Notch1 and Notch2 with siRNA [20], strongly indicating that the effect of Notch1 in these embryonic brain tumors is quite different from the oncogenic role we have observed in the astrocytic gliomas.

Because Notch signaling is versatile in different events occurring during embryogenesis, it is possible that the consequence of Notch activation depends on its normal function in a given tissue. Notch1 has recently been shown to promote the differentiation of various glial cell types, including Schwann cells in the peripheral nervous system, radial glia cells in the developing central nervous system, and Muller cells in the retina. It has been proposed that many radial glial cells develop into astrocytes. In contrast, Notch1 is not expressed in proliferating cerebellar precursors, but expressed in differentiated internal granular layer neurons, and Notch2 is expressed in the external granule cell layer of the developing cerebellum (rodent cerebellar granule cell precursors) and acts as a mitogen, and its expression negatively correlates with glial differentiation in mammalian brain development [5, 31–34]. Accordingly, whether the task performed by Notch receptors during development of the central nervous system is related to the differential expression and function of Notch1 and Notch2 in different types of brain tumors should be studied further.

It is speculated that although Notch signaling contributes to the process of tumorigenesis, collaborating molecular events are required for tumor formation. Notch signaling has been shown to interact with several signaling molecules and signaling pathways, including p53 [35], Ras [36, 37], NF-κB [38], Wnt [39, 40], Shh [41, 42], TGF-β [43], PI3K [44], and EGFR [45], which are important in tumorigenesis. Ras signaling is deregulated in most human cancers. Ras activates Notch signaling and Notch1 is necessary to maintain the malignant phenotype in Ras-transformed cells in vitro and in vivo [36, 37]. The interplays found between Notch and Wnt signaling and between Notch and Hedgehog (Shh) pathways are observed not only in development, but also in the pathogenesis of tumors, such as basal cell carcinoma and medulloblastoma [4, 25, 41, 42]. Recent evidence has demonstrated that activation of Notch signaling mediates the tumor-initiating effect of

TGF-α by inducing metaplastic conversion of differentiated pancreatic cells, indicating a direct link between EGFR activation and Notch signaling in the generation of pancreatic cancer precursors [45]. EGFR is known to play a key role in gliomagenesis, and our previous and current studies identify the crosstalk between EGFR and the Notch pathway in astrocytic gliomas. Knockdown of EGFR expression by RNA interference reduces Notch1 expression [23]. Targeting Notch1 with siRNA not only downregulates Notch1 expression but also reduces EGFR expression and its downstream signaling proteins, including PI3K, p-AKT, K-Ras, and cyclin D1, which are essential in cell proliferation and survival in gliomagenesis. It seems that there is a feedback loop or reciprocal interaction involving EGFR and Notch signaling. However, which is the initiator or primary event, or whether both are regulated by some other modulators during gliomagenesis remains to be elucidated further.

As shown in specimens of the tumors treated with Notch1 siRNA, downregulation of Notch1 also suppresses MMP9 and upregulates GFAP expression, suggesting that tumor cells are toward differentiation and invasive ability may be reduced. This result coincides with the study reported by Wang et al., in which they show that downregulation of Notch1 inhibits invasion of pancreatic cancer cells by inactivation of NF-κB, MMP9, and VEGF [46]. Cyclin D1 has been defined as a Notch target gene, and it is upregulated in activated Notch signaling. Stahl et al. have recently reported that Notch1 overexpression in rat kidney epithelial cells containing an estrogen-inducible activated Notch1 induces an increase of cyclin D1 mRNA level and transformation of cells [47]. This cell proliferation and transformation are prevented if cyclin D1 expression is inhibited with antisense cyclin D1 cDNA, indicating that it is an important downstream effector of Notch-induced cell transformation [47].

Furthermore, new evidence shows that Notch signaling in various tumor cells is able to activate endothelial cells and trigger angiogenesis in vitro and in xenograft mouse-tumor models, which might be one of the mechanisms for promoting tumor growth [48, 49]. Recent findings in *Drosophila melanogaster* indicated that constitutive activation of the Notch pathway or ectopic ligand Delta expression cooperating with the deregulation of Psq and Lola convert eye tissue overgrowth to tumors. The Notch pathway initiates the repressor of target gene *rbf*, just like its human counterpart RB1 gene, by epigenetic silencing of the promoter and transcription start region of *rbf*. This finding links the Notch pathway to the epigenetic silencing pathway in promoting tumorigenesis and may provide a new focus for studying Notch signaling [50]. Because Notch activation plays a role in the initiation and progression of many human malignancies, it is suggested that

Notch may be a novel cancer therapeutic target. It has been shown that γ-secretase inhibitors block Notch pathway-induced apoptosis and growth inhibition in Kaposi's sarcoma tumor cells with Notch activation [51], and it has also been reported that curcumin inhibits pancreatic cell growth and induces apoptosis by downregulation of Notch1 signaling [52].

Our findings in this study add to emerging evidence suggesting the oncogenic role of activated Notch1 in astrocytic gliomas and the complexity of the interplay and crosstalk between Notch and EGFR signaling pathways. The disparate effect of Notch1 in different types of brain tumor is a tissue-specific response to Notch signaling and the exact underlying mechanisms should be further investigated. Notch1 may be a potential therapeutic target for astrocytic gliomas.

References

1. Fleming RJ (1998) Structural conservation of Notch receptors and ligands. Semin Cell Dev Biol 9:599–607
2. Lewis J (1998) Notch signaling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol 9:583–589
3. Baron M (2003) An overview of the Notch signalling pathway. Semin Cell Dev Biol 14:113–119
4. Leong KG, Karsan A (2006) Recent insights into the role of Notch signaling in tumorigenesis. Blood 107:2223–2233
5. Hansson EM, Lendahl U, Chapman G (2004) Notch signaling in development and disease. Semin Cancer Biol 14:320–328
6. Iso T, Kedes L, Hamamori Y (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol 194:237–255
7. Lasky J, Wu H (2005) Notch signaling, brain development, and human disease. Pediatr Res 57:104R–109R
8. Miete L (2006) Notch signaling. Clin Cancer Res 12:1074–1079
9. Chiaramonte R, Basile A, Tassi E et al (2005) A wide role for Notch signaling in acute leukemia. Cancer Lett 219:113–120
10. Grabher C, von Boehmer H, Look AT (2006) Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukemia. Nat Rev Cancer 6:347–359
11. Stylianou S, Clarke RB, Brennan K (2006) Aberrant activation of Notch signaling in human breast cancer. Cancer Res 66:1517–1525
12. Pahlman S, Stockhausen MT, Fredlund E, Axelson H (2004) Notch signaling in neuroblastoma. Semin Cancer Biol 14:365–373
13. QI R, An H, Yu Y et al (2003) Notch 1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer Res 63:8323–8329
14. Sriuranpong V, Borges MV, Ravi RK, Arnold DR, Nelki BD, Baylin SB (2001) Notch signaling induces cell cycle arrest in small cell lung cancer cells. Cancer Res 61:3200–3205
15. Wang Z, Zhang Y, Li Y, Banerjee S, Liao J, Sarkar FH (2006) Downregulation of Notch 1 to cell growth inhibition and apoptosis in pancreatic cancer cells. Mol Cancer Ther 5:483–493
16. Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B (2002) Activated Notch 1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood 99:3398–3483
17. Axelson H (2004) Notch signaling and cancer: emerging complexity. Semin Cancer Biol 14:317–319
18. Purow BW, Haque RM, Noel MW et al (2005) Expression of Notch 1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 6596:2353–2363
19. Cuevas HC, Slocum AL, Jun P et al (2005) Meningioma transcript profiles reveal deregulated Notch signaling pathway. Cancer Res 65:5070–5076
20. Fan X, Mikolaenko I, Ethassan I et al (2004) Notch 1 and Notch 2 have opposite effects on embryonal brain tumor growth. Cancer Res 64:7787–7793
21. Jiang RC, Pu PY, Shen CH et al (2004) Preliminary study on cancer-related gene expression profiles in 63 cases of gliomas by cDNA array. Chin J Neurosurg 20:18–21
22. Pu P, Kang C, Zhang Z, Liu X, Jiang H (2006) Downregulation of PIK3CB by siRNA suppresses malignant glioma cell growth in vitro and in vivo. Technol Cancer Res Treat 5:271–280
23. Qiu M, Pu P, Kang C et al (2006) The experimental study on the downregulation of EGFR with siRNA inhibits the expression of Notch 1. Chin J Contemp Neurol Neurosurg 6:609–612
24. Maillard I, Pear WS (2003) Notch and cancer: best to avoid ups and downs. Cancer Cell 3:203–205
25. Radtke F, Raj K (2003) The role of Notch in tumorigenesis: oncogene or tumor suppressor? Nat Rev Cancer 3:756–767
26. Roy M, Pear WS, Aster JC (2007) The multifaceted role of Notch in cancer. Curr Opin Genet Dev 17:52–59
27. Kimura K, Satoh K, Kanno A et al (2007) Activation of Notch signaling in tumorigenesis of experimental pancreatic cancer induced by dimethylbenzanthracene in mice. Cancer Sci 98:155–162
28. Nicholas M, Wolfer A, Raj K, Kummer A (2003) Notch functions as a tumor suppressor in mouse skin. Nat Genet 33:416–421
29. Lefort K, Dotto GP (2004) Notch signaling in the integrated control of keratinocyte growth/differentiation and tumor suppression. Semin Cancer Biol 14:374–383
30. Weng AP, Aster JC (2004) Multiple niches in cancer: context is everything. Curr Opin Genet Dev 14:46–54
31. Tanaka M, Kadokawa Y, Hamada Y, Marunouchi T (1999) Notch 2 expression negatively correlates with glial differentiation in the postnatal mouse brain. J Neurobiol 41:524–539
32. Wang S, Barres BA (2000) Up a Notch: instructing gliogenesis. Neuron 27:197–200
33. Gaiano N, Nye JS, Fishell G (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron 26:395–404
34. Irvin DK, Zurcher SD, Nguyen T, Weinmaster G, Kornblum HI (2001) Expression patterns of Notch1, Notch2 and Notch3 suggest multiple functional roles for the Notch-CSL signaling system during brain development. J Comp Neurol 436:167–181
35. Sasaki Y, Ishida S, Moorimoto I et al (2002) The p53 family member genes are involved in the Notch signal pathway. J Biol Chem 277:710–724
36. Fitzgerald K, Harrington A, Leder P (2000) Ras pathway signals are required for Notch-mediated oncogenesis. Oncogene 19:4191–4198
37. Weijzen S, Rizzo P, Braid M et al (2002) Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 8:979–986
38. Ramdass B, Maliekal TT, Lakshmi S et al (2007) Coexpression of Notch1 and NF-kappa B signaling pathway components in human cervical cancer progression. Gynecol Oncol 104:352–361

39. Balint K, Xiao M, Pinnix CC et al (2005) Activation of Notch signaling is required for β-catenin-mediated primary melanoma progression. J Clin Invest 115:3166–3176

40. Deregowski V, Gazzerro E, Priest L, Rydziel S, Canalis E (2006) Notch1 overexpression inhibits osteoblastogenesis by suppressing Wnt/β-catenin but not bone morphogenetic protein signaling. J Biol Chem 281:6203–6210

41. Hallahan AR, Pritchard JI, Hansen S et al (2004) The SmoA1 mouse model reveals that Notch signaling is critical for the growth and survival of sonic Hedgehog-induced medulloblastomas. Cancer Res 64:7794–7800

42. Dakubo GD, Mazerolle CJ, Wallace VA (2006) Expression of Notch and Wnt pathway components and activation of Notch signaling in medulloblastomas from heterozygous patched mice. J Neurooncol 79:221–227

43. Niimi H, Pardali K, Vanlandewijck M, Heldin CH, Moustakas A (2007) Notch signaling is necessary for epithelial growth arrest by TGF-beta. J Cell Biol 176:695–707

44. McKenzie G, Ward G, Stallwood Y et al (2006) Cellular Notch responsiveness is defined by phosphoinositide-3-kinase-dependent signals. BMC cell Biol 7:10

45. Miyamoto Y, Maitra A, Ghosh B et al (2003) Notch mediates TGF-α-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell 3:565–576

46. Wang Z, Banerjee S, Li Y, Rahman KMW, Zhang Y, Sarkar FH (2006) Down-regulation of Notch1 inhibits invasion by inactivation of Nuclear Factor-kB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 66:2778–2784

47. Stahl M, Ge C, Shi S, Pestell RG, Stanley P (2006) Notch 1 induced transformation of RKE-1 cells requires up-regulation of cyclin D1. Cancer Res 66:7562–7570

48. Li JL, Harris AL (2005) Notch signaling from tumor cells: a new mechanism of angiogenesis. Cancer Cell 8:1–3

49. Rehman AO, Wang CY (2006) Notch signaling in the regulation of tumor angiogenesis. Trends Cell Biol 16:293–300

50. Dominguez M (2006) Interplay between Notch signaling and epigenetic silencers in cancer. Cancer Res 66:8931–8934

51. Miele L, Miao H, Nickoloff BJ (2006) Notch signaling as a novel cancer therapeutic target. Curr Cancer Drug Targets 6:313–323

52. Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH (2006) Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. Cancer 106:2503–2513