Receptors of the Notch signaling pathway are associated with hemorrhage of brain arteriovenous malformations

SHU LI[1], RONG WANG[1], YAJIE WANG[2], HAOWEN LI[2], JIAN ZHENG[1], RAN DUAN[1] and JIZONG ZHAO[1]

[1]Department of Neurosurgery; [2]Clinical Laboratory of Medical Research, Beijing Tiantan Hospital Affiliated to Capital Medical University, Beijing 100050, P.R. China

Received July 30, 2013; Accepted February 27, 2014

DOI: 10.3892/mmr.2014.2061

Abstract. Brain arteriovenous malformation (bAVM) is currently one of the most common cerebral vascular diseases, which result in severe clinical outcomes. The Notch signaling pathway is involved in vasculogenesis and angiogenesis, as well as vascular remodeling and arteriovenous differentiation in multiple diseases. Although there are previous studies on the correlation between bAVM and the Notch signaling pathway, none of these studies have elucidated whether abnormal expression levels of the key factors in this pathway are associated with hemorrhage of bAVMs. The present study compared the expression levels of *NOTCH1*, *NOTCH4* and two of their binding ligand genes, *DLL4* and *JAGGED1,* in bAVM nidus and normal superficial temporal arteries (STAs) by quantitative polymerase chain reaction and immunohistochemical staining. The bAVM patient group was further stratified into hemorrhage and non-hemorrhage groups to determine the expression levels of the four genes. It was observed that the expression levels of *NOTCH1* and *NOTCH4* were significantly increased in the bAVM cohort as compared with that of the control group. *DLL4* and *JAGGED1* exhibited the same expression levels in bAVMs and STAs. In addition, increased expression levels of *NOTCH1* were observed in the hemorrhage group compared with that of the non-hemorrhage group. However, the expression levels of *NOTCH4, DLL4* and *JAGGED1* showed no significant differences between the hemorrhage and non-hemorrhage groups. Abnormal *NOTCH1* expression was detected in the hemorrhage group, but other ligands of the Notch signaling pathway remained the same, suggesting that, although *NOTCH1* was upregulated in patients with bAVM, other ligands in this signaling pathway may be irrelevant to hemorrhage.

Correspondence to: Professor Jizong Zhao, Department of Neurosurgery, Beijing Tiantan Hospital Affiliated to Capital Medical University, 6 Tiantan Xili, Beijing 100050, P.R. China
E-mail: zhaojz205@gmail.com

Abbreviations: AVMs, arteriovenous malformations; PCR, polymerase chain reaction; STA, superficial temporal artery; MCA, middle cerebral artery; NICD, Notch intracellular domain; EC, endothelial cell

Key words: brain arteriovenous malformation, hemorrhage, Notch signaling pathway

Introduction

Brain arteriovenous malformations (bAVMs) were first described ~200 years ago and are defined as the direct communication of arteries to abnormally tortuous and dilated veins without interposing capillaries (1,2). bAVMs may present as hemorrhage, epilepsy, headache, non-hemorrhagic neurological deficits or are asymptomatic. Although it has been associated with certain well-defined genetic disorders, including ataxia telangiectasia, Osler-Weber-Rendu syndrome, Sturge-Weber syndrome and Wyburn-Mason syndrome (3-6), the molecular etiology of bAVM has not been fully elucidated. A previous study has suggested that bAVM may be congenital but dynamic with the possibility to grow, regress and even reappear (7). Several studies have found that there is an association between the upregulation of the Notch signaling pathway and bAVM (8-11). However, whether the pathway is involved in the hemorrhage of bAVMs, has yet to be fully elucidated.

The Notch signaling pathway is a transmembrane, evolutionarily conserved intercellular signaling pathway in vertebrates and non-vertebrates. It affects a wide variety of developmental processes and cell-fate determination during the embryonic period and postnatally (12-14). The Notch signaling pathway consists of four receptors (Notch1-4) and five ligands [Jagged 1 and 2, Delta-like (DLL) 1, 3 and 4] in mammals. It controls lymphatic endothelial quiescence, endothelial cell (EC) selection and the morphogenesis of vascular branches, as well as the interaction between the developing endothelium and endothelial basement membrane and arterial maturation (15-19). It has also been reported to coordinate with vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), the anaplastic lymphoma kinase signaling pathway, the Wnt/β-catenin pathway and nuclear factor-κB (16,17,20-23). The pathway is initiated with the binding of adjacent signaling cell ligands and receptors on receiving cells, followed by two proteolytic cleavage events. The second cleavage event releases the

Notch intracellular domain (NICD), which is then translocated to the nucleus and forms a complex with DNA-binding proteins, including CBF1, Su(H) and LAG-1(CSL), displaces previously bound co-repressors and recruits co-activators. In addition, the Notch signaling pathway functions as a transcriptional activator of the downstream targets, hairy and enhancer of split (Hes) and Hes-related protein gene families (24-26). Furthermore, the Notch pathway has been found to be associated with syndromes presenting with abnormal vascular phenotypes, including Alagille (27) and Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy syndromes (28,29).

The present study aimed to assess the expression levels of *NOTCH1, NOTCH4, DLL4* and *JAGGED1* in the nidus of patients with bAVM by quantitative polymerase chain reaction (qPCR) and immunohistochemical staining to determine whether there is an association between the Notch signaling pathway receptor or ligand expression levels and the clinical hemorrhage of bAVM.

Materials and methods

Subjects. A total of 35 nidus tissues from patients with bAVM were obtained from the Capital Medical University Affiliated Beijing Tiantan Hospital (Beijing, China) from June 2011 to June 2012. All patients were diagnosed with bAVM according to digital subtraction angiography and magnetic resonance imaging results combined with postoperative pathology diagnosis. The patients ranged in age from 7 to 57 years with a mean age of 29.29±14.64 years (median ± standard deviation). The majority of patients presented with the clinical symptoms of bAVM, including intracranial hemorrhage, epilepsy and headaches. Only one patient was recruited via regular medical examination. Among the 35 patients, five underwent endovascular treatment prior to surgery. The patients with bAVM were further divided into two groups: The hemorrhage group and the non-hemorrhage group, based on their first clinical presentation. The mean age of the hemorrhage and non-hemorrhage groups were 25.68±13.38 and 33.56±15.12 years, respectively. The clinical features of patients with bAVM are shown in Table I. Ten normal human superficial temporal arteries (STAs) were obtained from head trauma patients with a mean age of 45.7±16.3 years (range, 23-60 years). Informed consent was obtained from all patients and controls, either directly from the individual or their legal guardian. The protocol of the present study was approved by the ethics committee of the Beijing Tiantan Hospital (Beijing, China).

Table I. Clinical features of patients with brain arteriovenous malformation.

Clinical variables	Values
Age, years	30.10±14.97 (range, 8-57)
Gender, cases (%)	
Male	19 (54.29)
Female	16 (45.71)
Clinical presentation, cases (%)	
Epilepsy	17 (48.57)
Hemorrhage	16 (45.71)
Headache	5 (14.29)
Asymptomatic	1 (2.86)
Other cerebarl vascular diseases, cases	
Aneurysm	3
Ateriovenous fistula	1
Achnoid cyst	1
Prior embolism, cases (%)	5 (14.29)
S-M score	2.46±0.65

S-M score, Spetzler-Martin score. Values are expressed as the median ± standard deviation.

qPCR. Expression levels of mRNA were normalized against hypoxanthine phosphoribosyl-transferase-1 (HPRT-1), using the following specific PCR primers designed by the NCBI Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 2013); primers are shown in Table II. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and cDNA was acquired according to the Takara procedure (Takara Bio, Inc., Shiga, Japan) with 2 μg of total RNA. qPCR reactions for the four genes, *NOTCH1, NOTCH4, DLL4* and *JAGGED1*, were performed using the Takara Thermal Cycler Dice Real-Time System (TP800; Takara Bio, Inc.) and the conditions were as follows: 95˚C for 30 sec; 40 cycles comprising 95˚C for 5 sec, 55˚C for 30 sec, 72˚C for 30 sec; and a final hold at 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec. All reactions were performed in triplicate. After completion of qPCR, the amplification products underwent electrophoresis immediately on agarose gel. The target gene was confirmed according to the position in the DNA marker. Absorbance data were collected at the end of every extension, and cycle threshold (Ct) values were analyzed by the ΔCt method (30). The efficiency of amplification of the target genes and the internal control (HPRT-1) were examined.

Immunohistochemistry. Brain AVM specimens and STAs were embedded in paraffin and cut into 5-μm sections. Specimens were deparaffinized with xylene and rehydrated with ethanol following antigen retrieval with antigen unmasking solution according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 at room temperature for 10 min. Specimens were then washed with phosphate-buffered saline (PBS) and incubated in blocking solution for 3 min at a high pressure (pressure cooker; Biocare Medical, Inc., Concord, CA, USA). The primary antibodies used were as follows: Mouse monoclonal anti-Notch1 intracellular domain antibody (MAB5352, 1:300; Millipore, Billerica, MA, USA), rabbit polyclonal anti-Notch4 intracellular domain antibody (sc-5594, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-CD31 (ab28364, 1:200; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-Jagged-1 (ab7771, 1:200;

Table II. Primers designed for quantitative polymerase chain reaction.

Genes	Forward primers	Reverse primers
HPRT1	GCCCTGGCGTCGTGATTAGT	GGGCTACAATGTGATGGCCTCC
NOTCH1	GCCGCAGTTGTGCTCCTGAA	TGTCGTGATGCATGCGCTCC
NOTCH4	TGGAGAAGGGGCTGTGGAATG	CACACTGGCAGGTCCCTTGT
DLL4	CGCAATGACCACTTCGGCCA	ATTCGTTACACAGCCGGCCC
JAGGED1	TCGAGTCTGAGGCCGTTGCT	GAGACCGTGTCGGCTGCAAG

HPRT1, hypoxanthine phosphoribosyl-transferase-1; DLL, delta-like.

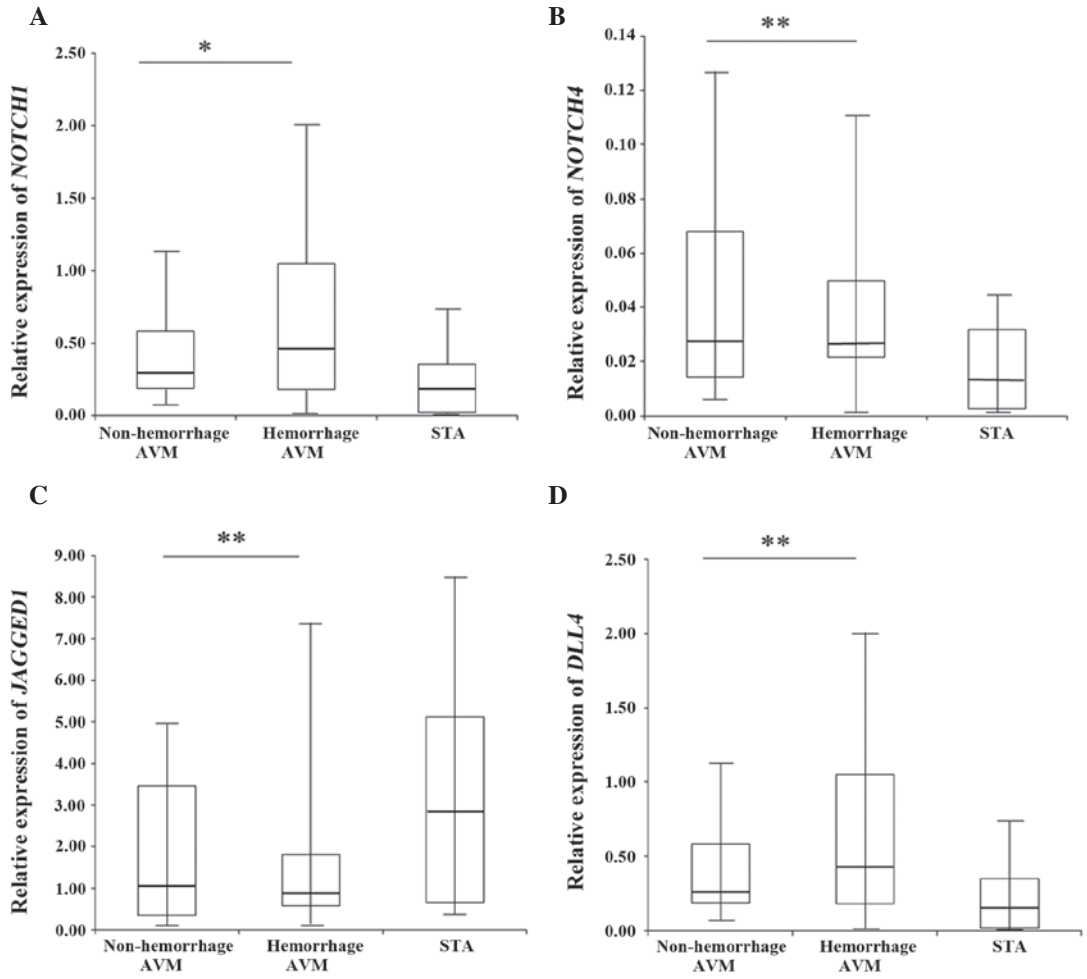

Figure 1. Relative mRNA expression levels of key factors of the Notch signaling pathway in hemorrhage AVM, non-hemorrhage AVM and STA. (A) *NOTCH1* expression levels increased by 2.82-fold in hemorrhaged AVM. (B) *NOTCH4* and (C) *DLL4* transcription levels remained unchanged. (D) *JAGGED1* was overexpressed in the non-hemorrhage group (1.24-fold), but without significance. $^{*}P<0.05$; $^{**}P>0.05$. AVM, arteriovenous malformation; STA, superficial temporal artery; DLL, delta-like.

Abcam), rabbit polyclonal anti-Hes-1 (ab71559, 1:100; Abcam) and rabbit polyclonal anti-DSL domain of DLL4 (ab7280, 1:150; Abcam). Primary antibodies were added to the blocking buffer and incubated with the tissue sections at 4˚C overnight. Sections were then washed with PBS and incubated with horseradish peroxidase-labeled goat anti-rabbit or anti-mouse antibody for 1 h at room temperature. 3,3'-diaminobenzidine solution (Vector Laboratories) was used to obtain a visible reaction product. The staining process was performed blind from the source. A Leica microscope and Leica digital color camera (DM4000; Leica, Mannheim, Germany) were used to examine and photograph the slides, respectively.

Statistical analyses. Statistical analysis was performed using a two sided t-test and one-way analysis of variance (ANOVA). All data are presented as the mean ± standard deviation. Data were analyzed using SPSS software, version 19.0 (SPSS Inc., Chicago, IL, USA). $P\leq0.05$ was considered to indicate a statistically significant difference.

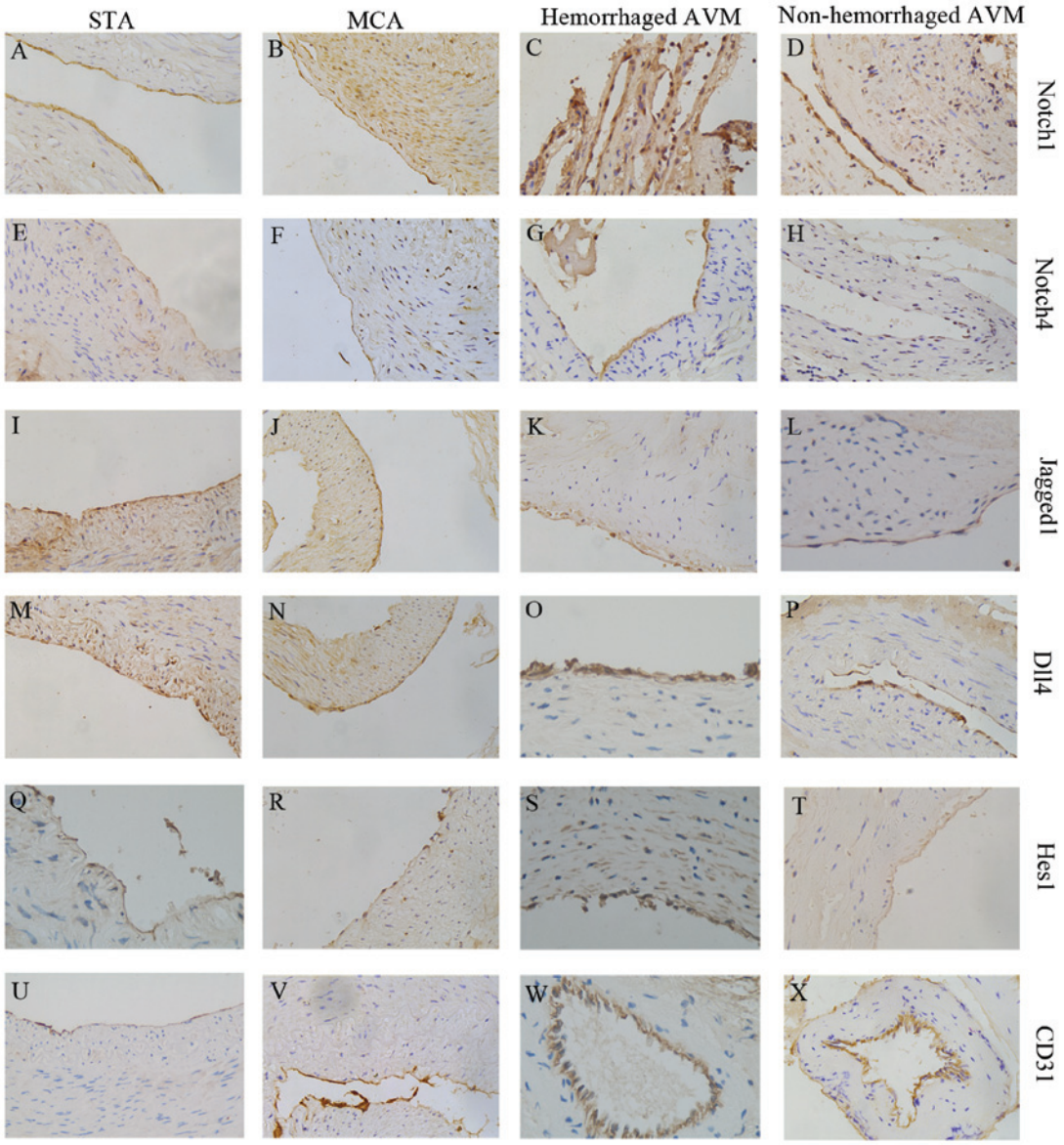

Figure 2. IH staining of STA, MCA, hemorrhaged AVM and non-hemorrhaged AVM samples. (A-D) Notch1, (E-H) in STA, MCA, hemorrhaged AVM and non-hemorrhaged AVM, respectively. (E-H) Notch4 in STA, MCA hemorrhaged AVM and non-hemorrhaged AVM. (I-L) Jagged1 in STA, MCA hemorrhaged AVM and non-hemorrhaged AVM. (M-P) IH stain for DLL4. (Q-T) IH stain for Hes1. There were no significant differences between the STA and MCA groups in (A,B) Notch1, (E,F) Notch4, (I,J) Jagged1, (M,N) DLL4, (Q,R) Hes1 and (U,V) CD31. In concordance with the quantitative polymerase chain reaction results, Notch1 and Notch4 expression were significantly increased in the AVM groups, whereas DLL4 and Jagged1 expression were similar in the STA control group. Compared with the hemorrhage bAVM and non-hemorrhage bAVM groups, only Notch1 was upregulated in hemorrhage brain AVM, other components remained unchanged in bAVM groups. (U-X) CD31 was stained to demonstrate endothelial cells. IH, immunohistochemical; STA, superficial temporal artery; MCA, middle cerebral artery; bAVM, brain arteriovenous malformation; DLL, delta-like; Hes-1, hairy and enhancer of split-1.

Results

Expression levels of the four genes in bAVM and STA specimens detected by immunohistochemistry. Elevated expression levels of *NOTCH1* and *NOTCH4* were detected in the hemorrhage bAVM group compared with that of the STA group. HPRT-1 was selected as the internal control for normalization. The expression levels of *NOTCH1* and *NOTCH4* were elevated by 2.26-fold (P=0.033) and 2.80-fold (P=0.002), respectively; however, the expression of *JAGGED1* and *DLL4* were slightly different but without significance (P=0.106 and P=0.492, respectively). In order to further examine the ligands involved in the Notch signaling pathway, immunohistochemical analysis was perfomed using a positive control, the middle cerebral artery (MCA). To determine the phenotype of expressing cells, anti-CD31 was selected as a representative. Immunohistological analysis demonstrated that Notch1 and Notch4 were weakly expressed in ECs in MCA and STA (Fig. 2), but overexpressed in the hemorrhage and non-hemorrhage groups. Jagged1 and DLL4 demonstrated similar expression levels in the two control and bAVM groups. The downstream target gene of the Notch signaling pathway, *HES1*, was predominantly expressed in nuclei of ECs and smooth muscle cells in bAVM, but was negatively expressed in the STAs and MCAs.

Expression levels of the four genes detected by qPCR. qPCR detected a significant increase (2.82-fold; 0.629±0.596) in

NOTCH1 expression in the hemorrhage group compared with that of the non-hemorrhage group (P=0.023). The expression levels of *NOTCH4* and *DLL4* remained unchanged between the two groups, while *JAGGED1* was overexpressed in the non-hemorrhage group (1.24-fold) (Fig. 1). However, the overexpression of *JAGGED1* lacked significance when analyzed by ANOVA. The expression levels detected by qPCR of the key components of the Notch signaling pathway in the bAVM groups were in concordance with the immunohistochemical results. The lack of significant differences in the expression of *JAGGED1* between the hemorrhage and non-hemorrhage groups indicated that the abnormal expression levels may be caused by the overall expression levels of *JAGGED1* in neural cells.

Discussion

bAVMs are commonly detected in patients aged ~30 years and there have been a number of cases reporting the regrowth of bAVM following complete surgery or embolism, reflecting the enigmatic etiology of bAVM (2,31). Due to the scarce data available on these cases, it is not possible to arbitrarily conclude that bAVM is a complete congenital lesion.

Previous studies have suggested that *NOTCH1* is an oncogene as its upregulation is associated with the presence of solid tumors in the digestive system, breast and hematological system (32-34). Uyttendaele *et al* (10) found that the introduction of the active form of *NOTCH4* and knockout of either *NOTCH1* or *NOTCH1*/*NOTCH4* in ECs produced similar phenotypes of vascular deficiency with the loss of fine branches or excessive branches. In addition, Murphy *et al* (9) observed the increased activation of endothelial Notch signaling with the presence of bAVM-like structures in mice, which suggested that the activation of the Notch signaling pathway is potentially a crucial molecular candidate in bAVM pathogenesis. In addition, Zhuge *et al* (11) identified the upregulation of Notch1 in human samples, confirming that abnormal Notch signaling activation has a vital role in the pathogenesis of bAVM. In a further study to elucidate the mechanisms of the Notch signaling pathway and determine potential treatment for bAVM, Murphy *et al* (9) focused on the normalization of the receptor Notch4. In his study, the high-flow AV shunts, caused by tetracycline-responsive system, decreased in blood flow as well as vessel diameter following repression of Notch4. Based on these findings, therapy that targets the Notch signaling pathway may be a promising approach for bAVM treatment (8,11,35). In addition, a previous study using mice demonstrated that a complete and safe normalization of Notch4 in animals reduced blood vessel size in bAVM, suggesting a treatment option of bAVM alternative to surgery, embolism or radiation therapy (8,35).

In the present study, elevated expression levels of *NOTCH1* and *NOTCH4* were found in the bAVM nidus at the RNA and protein levels compared with those of normal STA and normal MCA, which was in accordance with previous research (10,11,35). To the best of our knowledge, the present study was the first to determine whether the Notch signaling pathway is associated with the clinical presentation of hemorrhage in bAVM. Patients with bAVM were stratified into the hemorrhage and non-hemorrhage groups and the expression levels of key factors in the Notch signaling pathway were compared between the groups. It was found that the expression levels of *NOTCH1* significantly increased in the hemorrhage group, suggesting that *NOTCH4, DLL4* and *JAGGGED1* may not be involved in the pathogenesis of hemorrhage in bAVM nidus. Recently, Liebler *et al* (36) reported that while Notch1 NICD markedly repressed endothelial migration and sprouting angiogenesis, the intracellular domain of Jagged1 and DLL4 did not influence EC adhesion. It is therefore concluded that the relevance of ligand intracellular domains in ECs remains unclear. However, our findings also showed different results compared with those of Zhuge *et al* (11). It was demonstrated that the expression levels of DLL4 and JAGGED1 appeared unchanged between patients with bAVM and the controls. As a large sample size was used to contribute to the power of detection, the data are considered reliable.

In the present study, it was demonstrated that the expression levels of *NOTCH1* and *NOTCH4* were increased in patients with bAVM, and the correlation between hemorrhage and Notch1/4 expression was analyzed. It was demonstrated that the increased expression of key components of the Notch signaling pathway are a potential cause of hemorrhage in human bAVM. The findings also suggested that, although the continuous activation of the Notch signaling pathway was reported to lead to hemorrhage in the brain, liver or other organs in studies of mice and embryos (8,9), there was no significant association between hemorrhage of bAVM and Notch4, DLL4 and Jagged1; however, there was an association between hemorrhage and Notch1. Further studies targeting the remaining receptors and ligands of the Notch signaling pathway are required to conclude whether Notch1 is the key factor involved in hemorrhage in bAVM. This would elucidate the importance of anti-Notch signaling drugs and their efficiency to reduce the risk of hemorrhage in patients with bAVM.

References

1. Porter AJ and Bull J: Some aspects of the natural history of cerebral arteriovenous malformation. Br J Radiol 42: 667-675, 1969.
2. Gross BA and Du R: Natural history of cerebral arteriovenous malformations: a meta-analysis. J Neurosurg 118: 437-443, 2013.
3. Nussbaum ES, Heros RC, Madison MT, Awasthi D and Truwit CL: The pathogenesis of arteriovenous malformations: insights provided by a case of multiple arteriovenous malformations developing in relation to a developmental venous anomaly. Neurosurgery 43: 347-351, 1998.
4. Ponce FA, Han PP, Spetzler RF, Canady A and Feiz-Erfan I: Associated arteriovenous malformation of the orbit and brain: a case of Wyburn-Mason syndrome without retinal involvement. Case report. J Neurosurg 95: 346-349, 2001.
5. Stapf C, Mohr JP, Pile-Spellman J, Solomon RA, Sacco RL and Connolly EJ Jr: Epidemiology and natural history of arteriovenous malformations. Neurosurg Focus 11: e1, 2001.
6. Wohlwill FJ and Yakovlev PI: Histopathology of meningo-facial angiomatosis (Sturge-Weber's disease); report of four cases. J Neuropathol Exp Neurol 16: 341-364, 1957.
7. Sturiale CL, Puca A and Sebastiani P, *et al*: Single nucleotide polymorphisms associated with sporadic brain arteriovenous malformations: where do we stand? Brain 136: 665-681, 2013.
8. Murphy PA, Kim TN, Lu G, Bollen AW, Schaffer CB and Wang RA: Notch4 normalization reduces blood vessel size in arteriovenous malformations. Sci Transl Med 4: 117r-118r, 2012.
9. Murphy PA, Lam MT, Wu X, *et al*: Endothelial Notch4 signaling induces hallmarks of brain arteriovenous malformations in mice. Proc Natl Acad Sci USA 105: 10901-10906, 2008.

10. Uyttendaele H, Ho J, Rossant J and Kitajewski J: Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proc Natl Acad Sci U S A 98: 5643-5648, 2001.
11. ZhuGe Q, Zhong M and Zheng W, *et al*: Notch-1 signalling is activated in brain arteriovenous malformations in humans. Brain 132: 3231-3241, 2009.
12. Irvine KD: Fringe, Notch, and making developmental boundaries. Curr Opin Genet Dev 9: 434-441, 1999.
13. Li JL and Harris AL: Notch signaling from tumor cells: a new mechanism of angiogenesis. Cancer Cell 8: 1-3, 2005.
14. Muskavitch MA: Delta-notch signaling and Drosophila cell fate choice. Dev Biol 166: 415-430, 1994.
15. Alberi L, Hoey SE, Brai E, Scotti AL and Marathe S: Notch signaling in the brain: in good and bad times. Ageing Res Rev 12: 801-814, 2013.
16. Holderfield MT and Hughes CC: Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. Circ Res 102: 637-652, 2008.
17. Lawson ND, Scheer N, Pham VN, *et al*: Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 128: 3675-3683, 2001.
18. Weng AP, Ferrando AA, Lee W, *et al*: Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306: 269-271, 2004.
19. Zheng W, Tammela T, Yamamoto M, *et al*: Notch restricts lymphatic vessel sprouting induced by vascular endothelial growth factor. Blood 118: 1154-1162, 2011.
20. Clément N, Gueguen M, Glorian M, *et al*: Notch3 and IL-1beta exert opposing effects on a vascular smooth muscle cell inflammatory pathway in which NF-kappaB drives crosstalk. J Cell Sci 120: 3352-3361, 2007.
21. Corada M, Nyqvist D, Orsenigo F, *et al*: The Wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating Dll4/Notch signaling. Dev Cell 18: 938-949, 2010.
22. Domigan CK and Iruela-Arispe ML: Recent advances in vascular development. Curr Opin Hematol 19: 176-183, 2012.
23. Larrivée B, Prahst C, Gordon E, *et al*: ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. Dev Cell 22: 489-500, 2012.
24. Lasky JL and Wu H: Notch signaling, brain development, and human disease. Pediatr Res 57: 104R-109R, 2005.
25. Bray SJ: Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7: 678-689, 2006.
26. Roca C and Adams RH: Regulation of vascular morphogenesis by Notch signaling. Genes Dev 21: 2511-2524, 2007.
27. Rocha R, Soro I, Leitão A, Silva ML and Leão M: Moyamoya vascular pattern in Alagille syndrome. Pediatr Neurol 47: 125-128, 2012.
28. Joutel A, Corpechot C, Ducros A, *et al*: Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 383: 707-710, 1996.
29. Louvi A and Artavanis-Tsakonas S: Notch and disease: a growing field. Semin Cell Dev Biol 23: 473-480, 2012.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
31. Halim AX, Johnston SC, Singh V, *et al*: Longitudinal risk of intracranial hemorrhage in patients with arteriovenous malformation of the brain within a defined population. Stroke 35: 1697-1702, 2004.
32. Reedijk M, Odorcic S, Chang L, *et al*: High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res 65: 8530-8537, 2005.
33. Weijzen S, Rizzo P, Braid M, *et al*: Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nat Med 8: 979-986, 2002.
34. Sun Y, Gao X, Liu J, *et al*: Differential Notch1 and Notch2 expression and frequent activation of Notch signaling in gastric cancers. Arch Pathol Lab Med 135: 451-458, 2011.
35. Murphy PA, Lu G, Shiah S, Bollen AW and Wang RA: Endothelial Notch signaling is upregulated in human brain arteriovenous malformations and a mouse model of the disease. Lab Invest 89: 971-982, 2009.
36. Liebler SS, Feldner A, Adam MG, Korff T, Augustin HG and Fischer A: No evidence for a functional role of bi-directional Notch signaling during angiogenesis. PLoS One 7: e53074, 2012.