Involvement of TLR2/4-MyD88-NF-κB signaling pathway in the pathogenesis of intracranial aneurysm

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Abstract. Toll-like receptor (TLR) 2/4 serves an important regulatory role in nerve tissue injury. However, the downstream and potential mechanisms remain to be elucidated. The present study was designed to investigate the roles of the TLR2/4-major myeloid differentiation response gene 88 (MyD88)-NF-κB signaling pathway in the development of intracranial aneurysm. The expression of TLR2, TLR4 and MyD88 in the blood of normal controls and patients with intracranial aneurysm were detected by quantitative PCR and ELISA. Human brain vascular smooth muscle cells were treated by Angiotensin II (Ang II) to evaluate the involvement of TLR2/4-MyD88-NF-κB signaling pathway in the process. The *in vitro* experiment was divided into four groups: The control group, an Ang II group, an Ang II + small interfering (si)RNA control group and an Ang II + TLR2-group. Cell viability, migration, apoptosis and expression of TLR2, TLR4, MyD88, NF-κB and phosphorylated (p-)p65 expression were detected. The results demonstrated that the expression of TLR2, TLR4, MyD88 and NF-κB at mRNA and protein levels in patients with intracranial aneurysm was significantly higher compared with corresponding protein in normal controls ($P<0.05$). *In vitro* experiments demonstrated that Ang II treatment increased the cell proliferation and migration rate but reduced the apoptotic rate compared with the control ($P<0.05$). The expression of TLR2, TLR4, MyD88, NF-κB and p-p65 was significantly increased in the Ang II group (vs. control; $P<0.05$). By contrast, TLR2-short interfering RNA reduced the cell proliferation and migration rate, and reduced the expression of TLR2, TLR4, MyD88, NF-κB and p-p65 (vs. Ang II + short interfering RNA control; $P<0.05$). In conclusion, the data of the present study indicated that the TLR2/4-MyD88-NF-κB signaling pathway is involved in the pathogenesis of intracranial aneurysm.

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Introduction

Intracranial aneurysm is one of the common disorders in clinical neuroscience with an incidence of approximately 3.2% worldwide (1). The incidence of intracranial aneurysm in the female population is at ≤12.9%, significantly higher compared with the male population (2). Notably, intracranial aneurysm is a non-ignorable factor for subarachnoid hemorrhage following cerebral thrombosis and hypertensive cerebral hemorrhage in cerebrovascular accidents (3,4). Indeed, >85% of spontaneous subarachnoid hemorrhage is caused by rupture of aneurysms (3,4). Therefore, it is of great clinical significance to explore the pathogenesis of intracranial aneurysms and find effective therapeutic targets.

There are many types of Toll-like receptor (TLRs) subfamilies. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are found on the cell surface and can be activated by a variety of extracellular ligands, while TLR3, TLR7, TLR8 and TLR9 are intracellularly expressed and generally recognized by nucleic acid structure (5). TLRs serve important roles in the inflammatory response of central nervous system (6). TLR2 and TLR4 expression increases in patients with ischemic stroke and TLR4 knockdown attenuates brain edema, inflammation and damage after intracerebral hemorrhage (ICH) (7). In addition, TLR2 promotes inflammatory damage after ICH (8). Hemoglobin can induce the formation of TLR2/TLR4 heterodimer after ICH, which can amplify the harmful effects of TLR2 and TLR4 in the brain (9).

The major myeloid differentiation response gene 88 (MyD88) is an important adaptor that is downstream of TLR2 and TLR4 (10). Activated MyD88 can promote the phosphorylation and nuclear translocation of NF-κB, thus upregulating the expression of pro-inflammatory factors and matrix metalloproteinases and aggravating tissue damage (11-13). Therefore, TLR2/4 and its downstream signaling pathway serve an important regulatory role in nerve tissue injury. It has also been reported that TLR4 is upregulated during cerebral aneurysm formation in endothelial cells (14,15). How TLR2/4 and their downstream signaling pathway is involved in the pathogenesis of intracranial aneurysm remains to be elucidated.

The present study focused on the association between the TLR2/4-MyD88-NF-κB signaling pathway and intracranial aneurysm. The expression of the TLR2/4-MyD88-NF-κB signaling pathway was detected in the serum from intracranial aneurysm patients. Through an *in vitro* model, the involvement

of the TLR2/4-MyD88-NF-κB signaling pathway in the pathogenesis of intracranial aneurysm was investigated.

Materials and methods

Patients. Venous blood (5 ml; upper extremity) was collected from patients with intracranial aneurysm (n=222) and age- and sex-matched normal controls (n=200). The inclusion criteria were as follows: Intracranial aneurysm diagnosed by digital subtraction angiography following initial screening of magnetic resonance angiogram or computerized tomography angiography; ruptured aneurysm; Hunt and Hess grade 1-3 (16); and patients receiving endovascular embolization. The exclusion criteria included: Delayed neurological deficits; Glasgow Coma Scale score (17) decreased by ≥2 points; with or without cerebral infarction unrelated to aneurysm treatment or other causes; neurogenic pulmonary edema, hydrocephalus and epilepsy. In total, 222 patients (50% male; age: 45-65 years) were enrolled and 200 normal controls (50% male; age: 44-65 years) were recruited from the Health Examination Center of the First Affiliated Hospital of Nanchang University between January 1, 2018 and May 1, 2019. Written informed consents were obtained from the participants. All experimental procedures were approved by the Ethics Committee of Nanchang University (approval no. NCU-20201213).

Reverse transcription-quantitative (RT-q)PCR. Total RNA from blood cells ($3x10^5$ cells/ml) was extracted using a TRIzol® assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The purity of RNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) based on the optical density (OD): OD280/260. Thereafter, RNA was transcribed into cDNA according to the instructions of the reverse transcription kit (cat. no. 639522; Takara Biotechnology Co., Ltd.). qPCR was used to detect the expression level of the targeted genes using the synthesized cDNA as templates. The primers (5'→3') are listed in Table I. The PCR was processed in a 20 μl reaction system including 1 μl cDNA, 2 μl primers, 10 μl 2X ULtraSYBR Mixture (CoWin Biosciences) and 7 μl dH_2O with thermocycling as follows: Denaturing 10 sec at 95˚C, annealing 30 sec at 58˚C and extension 30 sec at 72˚C for 40 cycles. Relevant expression of MyD88, TLR2 and TLR4 was normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method (18). Experiments were repeated 6 times.

ELISA. TLR2 (cat. no. ab131556; Abcam), TLR4 (cat. no. MBS2702401; MyBioSource, Inc.), MyD88 (cat. no. ab171341; Abcam) and NF-κB (cat. no. ab133112; Abcam) levels in serum were detected by ELISA method following the instructions of the assay kits.

Experimental groups. Human brain vascular smooth muscle cells (BVSMCs) were purchased from Shanghai Maisha Biotechnology Co., Ltd, and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin in 5% CO_2 at 37˚C. The experiments were divided into four groups: A control group, an angiotensin II (Ang II) group, an Ang II + small interfering (si)RNA control group and an Ang II + TLR2-siRNA group. The plasmids were transfected when cell confluence reached 80%. The transfection solution included 125 μl optiMEM, 5 μl Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) and 2.5 μg pLV Puro vector (cat. no. VL3103; Beijing Inovogen Tech. Co., Ltd.) or 1 μg siRNA, and the mixture was incubated at room temperature for 5 min. The mixture was added into the corresponding well (final concentration of plasmid: 1 μg/ml). The culture medium (medium containing 20% FBS) was refreshed 6 h following transfection. Following 24 h of transfection, BVSMCs were treated with Ang II to induce cell remodeling as previously described (19). The sequences of three TLR2 siRNAs and negative control (NC) were synthesized by Universal Biological Systems (Anhui) Co., Ltd. and were as follows: TLR2 siRNA-1, 5'-GCUGACAUC CAAUGGAAUUAAUUCCAUUGGAUGUCAGC-3'; TLR2 siRNA-2, 5'-GGGACUUCAUUCCUGGCAAUUGCCAGG AAUGAAGUCCC-3'; TLR2 siRNA-3, 5'-GCAAGCUGC GGAAGAUAAUAUUAUCUUCCGCAGCUUGC-3'; and NC, 5'-UUCUCCGAACGUGUCACGUTTACGUGACACGUUCG GAGAATT-3'.

Following treatment for 48 h, cell remodeling of BVSMCs was detected based on cell proliferation, migration and apoptosis. The expressions of TLR2, TLR4, MyD88, NF-κB and p-p65 were detected by RT-qPCR and western blotting.

Cell Counting Kit-8 (CCK-8) assay. Medium (10 μl) with CCK-8 (Gibco; Thermo Fisher Scientific, Inc.) was added into each well following drug treatment or transfection. After an additional 4-h incubation in a CO_2 incubator at 37˚C, the absorbance at 570 nm was recorded by a microplate reader (Thermo Fisher Scientific, Inc.). Cell proliferation was calculated based on OD values.

Flow cytometry. The cells in each group were collected after digestion by trypsin (Gibco; Thermo Fisher Scientific, Inc.). Thereafter, the cells were incubated with Annexin V-FITC and propidium iodide using an Annexin V-FITC apoptosis detection kit (cat. no. C1062; Beyotime Institute of Biotechnology) for 30 min in the dark at room temperature. Apoptosis was detected using an Accuri C6 flow cytometer (BD Biosciences) within 1 h. Data were analyzed using FlowJo software (version 7.6; FlowJo, LLC). The apoptotic rate was calculated based on the percentages of early apoptotic cells (EA) and late apoptotic cells (LA). The equation was as follows: Apoptotic rate (%) = [(Number of EA + Number of LA)/Total number of EA and LA] x100.

Cell migration. Cells were seeded into a 6-well plate ($1x10^6$ cells/well) and following 24 h of incubation at 37˚C, a uniform line was made across the center of the well using a 200-μl pipette tip. Following 24 or 48 h incubation in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS in a CO_2 incubator at 37˚C, the images were captured under a light microscope (magnification, x200; Olympus Corporation). The migration speed was calculated based on the following formula: Cell mobility (μm/h) = [$Width_{(1)}$-$width_{(2)}$]/72 h. $Width_{(1)}$ represented the width measured immediately (0 h) after drawing the line. $Width_{(2)}$ represented the width measured 24 or 48 h after drawing the line.

Western blotting. Protein was extracted by a protein isolation kit (cat. no. 28-9425-44; ReadyPrep) which was purchased from Cytiva. The concentration of the protein was quantified with a bicinchoninic acid protein assay kit. Thereafter, 25 μg protein was

Table I. Primer sequences of MyD88, TLR2 and TLR4.

Genes	Sequence (5'→3')	Primer length (bp)	Product length (bp)	Annealing temperature (˚C)
MyD88 F	CAGCGACATCCAGTTTGTGC	20	153	59.7
MyD88 R	GGCCTTCTAGCCAACCTCTT	20		
TLR2 F	ATGCTGCCATTCTCATTCTTC	21	100	57.8
TLR2 R	TCCAGGTAGGTCTTGGTGTTC	21		
TLR4 F	GACCTGTCCCTGAACCCTAT	20	136	57.6
TLR4 R	CTAAACCAGCCAGACCTTGA	20		
GAPDH F	CAATGACCCCTTCATTGACC	20	106	57.2
GAPDH R	GAGAAGCTTCCCGTTCTCAG	20		

MyD88, major myeloid differentiation response gene 88; TLR, Toll-like receptor; F, forward; R, reverse.

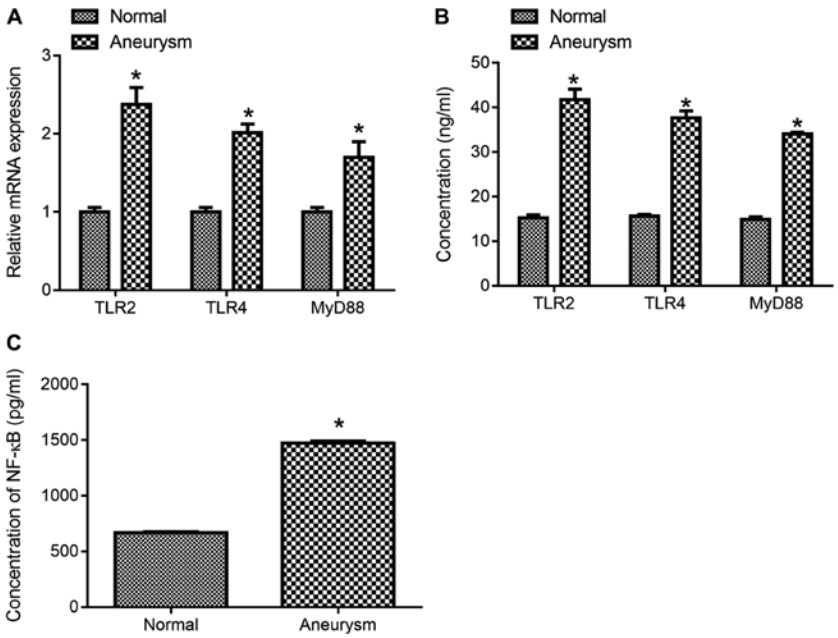

Figure 1. Expression of TLR2, TLR4, MyD88 and NF-κB p65 is promoted in patients with intracranial aneurysms. (A) TLR2, TLR4 and MyD88 expression at mRNA level; (B) TLR2, TLR4 and MyD88 expression at protein level; and (C) NF-κB p65 protein level. *P<0.05 vs. normal control (normal control: n=200; patients: n=222). TLR, Toll-like receptor; MyD88, major myeloid differentiation response gene 88.

separated via 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were then transferred onto PVDF membranes and blocked in 5% milk (2 h at room temperature). The following primary antibodies were incubated with the membranes overnight at 4˚C: Rabbit polyclonal anti-TLR2 (1:500; cat. no. ab213676, Abcam); rabbit polyclonal anti-TLR4 (1:1,000; cat. no. bs-20594R, BIOSS); rabbit polyclonal anti-MyD88 (1:1,000; cat. no. bs-1047R, BIOSS); rabbit polyclonal anti-NF-κB p65 (1:1,000; cat. no. bs-0465R, BIOSS); rabbit polyclonal anti-p-p65 (1:1,000; AF2006, affinity). The secondary antibody (1:2,000; ZB-2305; OriGene Technologies, Inc.) was co-incubated for 2 h at room temperature. Protein bands were visualized using ECL solution (cat. no. SW2010-1; Beijing Solarbio Science & Technology Co., Ltd.). Densitometric analysis was performed using Quantity One software (version 4.6; Bio-Rad Laboratories, Inc.).

Statistical analyses. Data in the present study were presented as the mean ± standard deviation and analyzed using SPSS version 17.0 (SPSS, Inc.). All data were in normal distribution and unpaired t-test or one-way analysis of variance followed by the Bonferroni's test was employed in the data analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of TLR2, TLR4, MyD88 and NF-κB in the serum of patients with intracranial aneurysms. The expression of TLR2, TLR4 and MyD88 mRNA in patients with intracranial aneurysm was significantly higher compared with normal controls (Fig. 1A; P<0.05). The expression levels of TLR2, TLR4, MyD88 (Fig. 1B) and NF-κB p65 (Fig. 1C) in patients with intracranial aneurysm were significantly higher compared with normal control (Fig. 1B and C; P<0.05).

TLR2-siRNA attenuates Ang II-induced cell proliferation of BVSMCs. As shown in Fig. 2A and B, 10^{-7} M Ang II facilitated

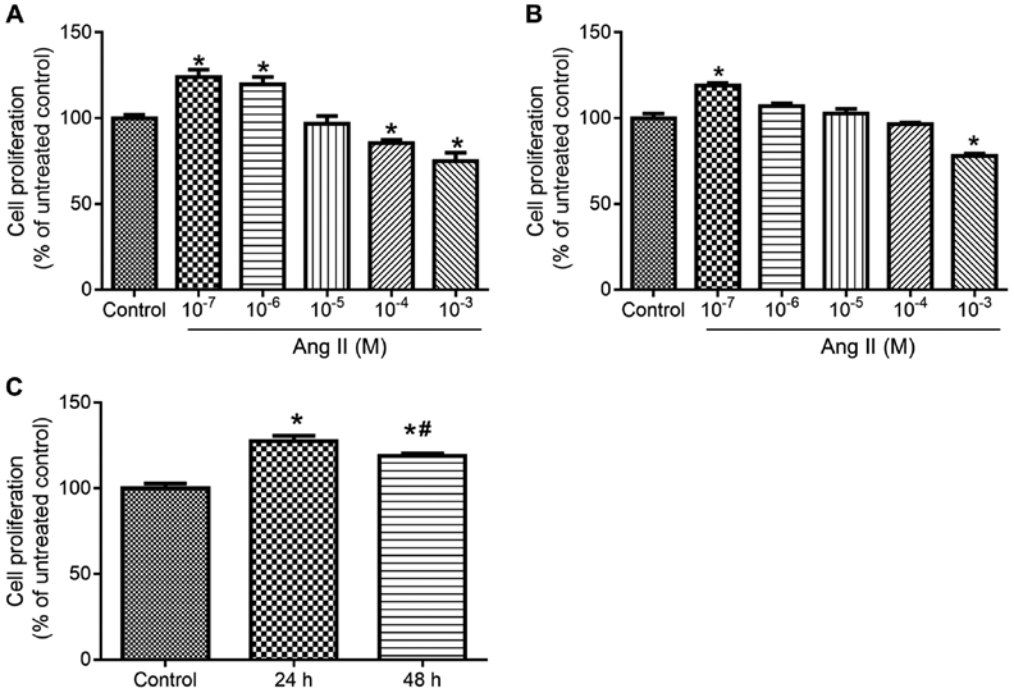

Figure 2. Ang II promotes the cell proliferation of human brain vascular smooth muscle cells. (A) Ang II treatment for 24 h; (B) Ang II treatment for 48 h; and (C) Treatment by 10^{-7} M Ang II for 24 or 48 h. *P<0.05 vs. control group, #P<0.05 vs. 24 h group (n=6). Ang II, Angiotensin II.

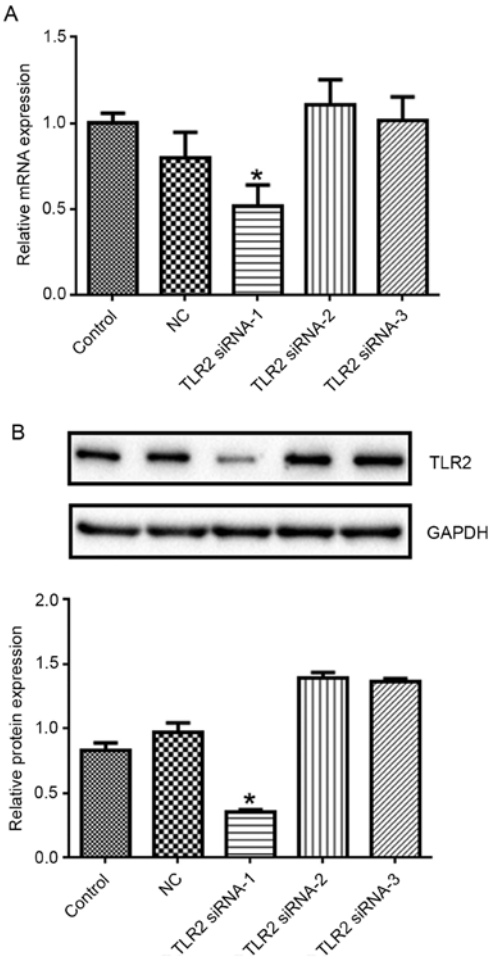

Figure 3. TLR2-siRNA reduces TLR2 expression in human brain vascular smooth muscle cells. (A) TLR2 expression at mRNA level and (B) TLR2 expression at protein level. *P<0.05 vs. NC (n=6). TLR, Toll-like receptor; si, small interfering; NC, negative control.

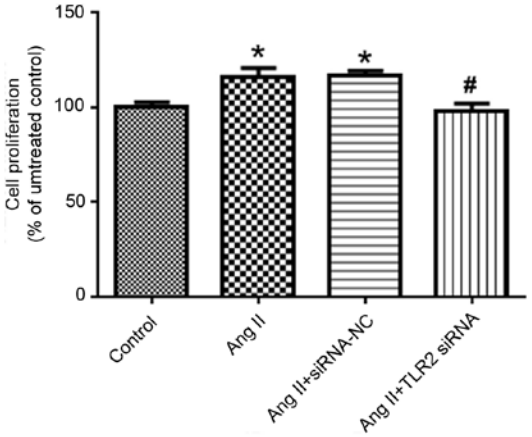

Figure 4. TLR2-siRNA attenuates Ang II-induced cell proliferation. Compared with, *P<0.05 vs. control group; #P<0.05 vs. Ang II + siRNA-NC (n=6). TLR, Toll-like receptor; si, small interfering; Ang II, Angiotensin II; NC, negative control.

cell proliferation after administration for 24 and 48 h compared with control group. Thus, this concentration of Ang II was selected to produce the remodeling model of BVSMCs. As shown in Fig. 2C, the cell proliferation increased significantly 24 h after Ang II administration. Therefore, 10^{-7} M Ang II was selected to treat the cells for 24 h.

TLR2 expression in the TLR2 siRNA-1 group decreased significantly (Fig. 3; P<0.05 vs. control). Therefore, TLR2 siRNA-1 was selected in the subsequent experiments.

Compared with the control group, the proliferation of Ang II group increased significantly (P<0.05; Fig. 4). By contrast, the cell proliferation of Ang II + TLR2 siRNA group decreased significantly (P<0.05 vs. Ang II + siRNA NC group).

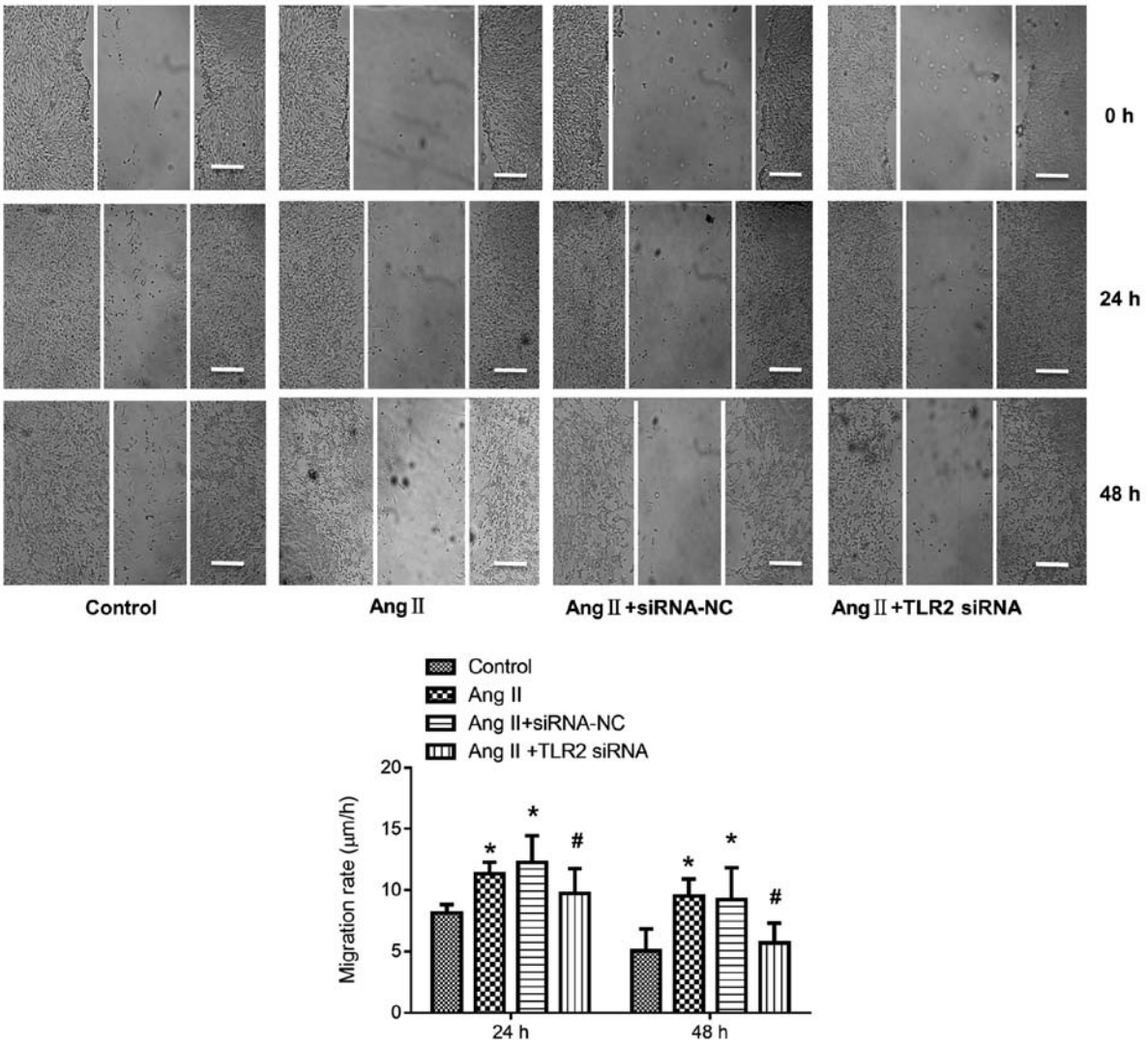

Figure 5. TLR2-siRNA attenuates Ang II-induced cell migration. Upper panel: Representative images of the cells (scale bar, 100-μm); lower panel: Quantification data of cell migration rate. *P<0.05 vs. control group; #P<0.05 vs. Ang II + siRNA-NC (n=6). TLR, Toll-like receptor; si, small interfering; Ang II, Angiotensin II; NC, negative control.

TLR2-siRNA attenuates Ang II-induced cell migration. Compared with control group, the cell migration rate of Ang II group increased significantly (P<0.05). By contrast, the cell migration rate of Ang II + TLR2 siRNA group decreased significantly compared with Ang II + siRNA NC group (P<0.05; Fig. 5).

TLR2-siRNA attenuates Ang II-induced decrease of apoptosis of BVSMCs. Compared with the control group, the apoptosis rate of Ang II group decreased significantly (P<0.05). By contrast, the apoptosis rate of Ang II + TLR2 siRNA group increased significantly (Fig. 6; P<0.05 vs. Ang II + siRNA NC group).

TLR2-siRNA reduced Ang II-induced expression of TLR2, TLR4 and MyD88. Compared with control group, TLR2, TLR4 and MyD88 expression at mRNA and protein levels in Ang II group increased significantly (P<0.05). Compared with Ang II + siRNA NC group, TLR2, TLR4 and MyD88 expression in Ang II + TLR2 siRNA group decreased significantly (P<0.05; Fig. 7).

TLR2-siRNA attenuates Ang II-induced expression of NF-κB p65 and p-p65 of BVSMCs. Compared with control group, the expression of NF-κB p65, p-p65 and p-p65/p65 in the Ang II group was significantly higher (P<0.05; Fig. 8). Compared with Ang II + siRNA NC group, the expression of NF-κB p65, p-p65 and p-p65/p65 in Ang II + TLR2 siRNA group was significantly lower (P<0.05; Fig. 8).

Discussion

The present study reported that the expression of TLR2/4-MyD88-NF-κB was promoted in patients with intracranial aneurysm. *In vitro* experiments revealed that Ang II-induced remodeling of BVSMCs was ameliorated by TLR2 silencing. Clinical evidence and results from basic experiments revealed that TLR2/4-MyD88-NF-κB signaling pathway was potentially involved in the development of intracranial aneurysm.

The pathogenesis of intracranial aneurysm remains to be fully elucidated, although genetic, environmental, estrogen level, hypertension, smoking and alcohol are closely

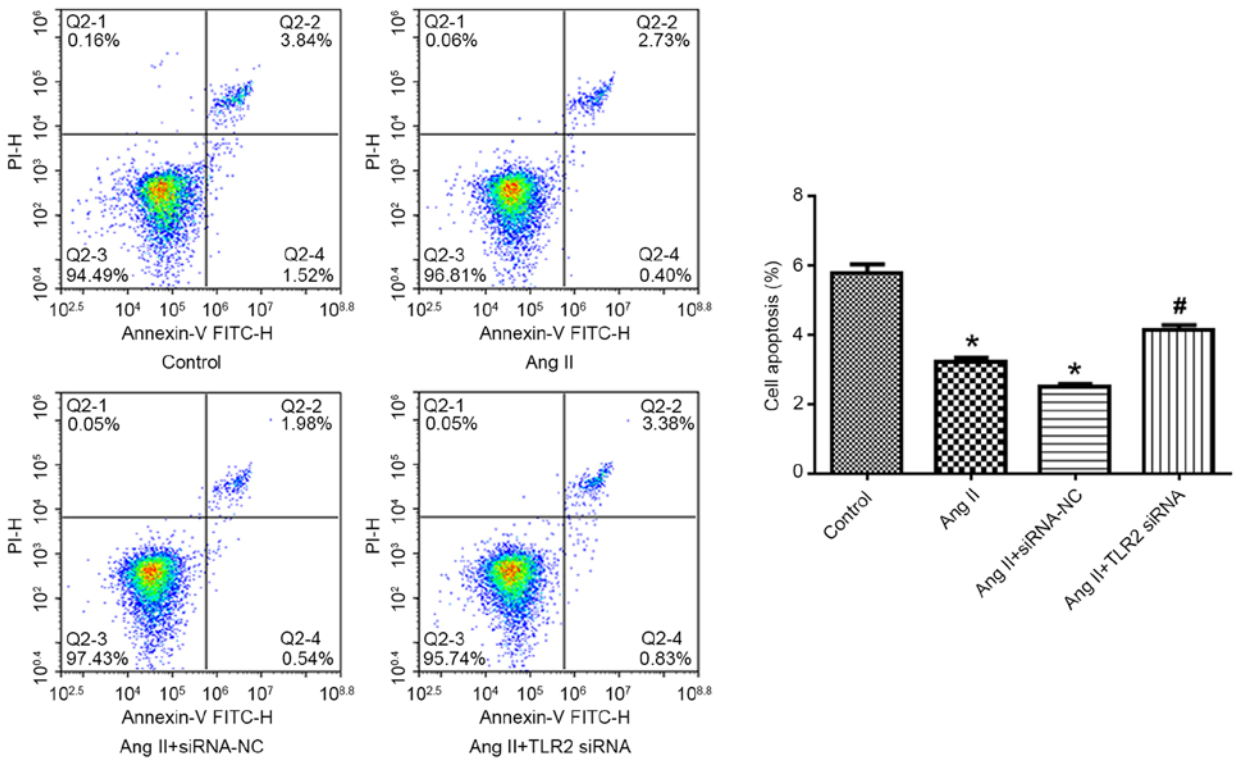

Figure 6. TLR2-siRNA attenuates Ang II-induced decrease of apoptosis. Left panel: Representative images of flow cytometry; right panel: Quantification data of apoptosis. *P<0.05 vs. control group; #P<0.05 vs. Ang II + siRNA-NC (n=6). TLR, Toll-like receptor; si, small interfering; Ang II, Angiotensin II; NC, negative control.

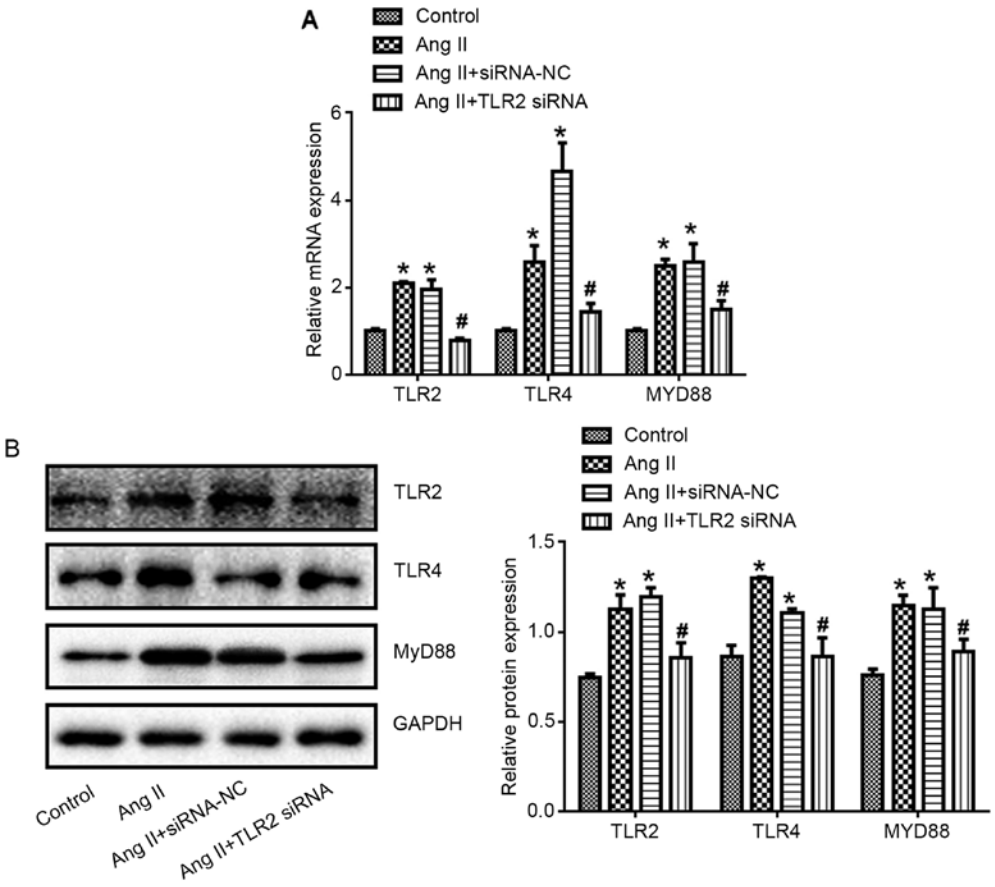

Figure 7. TLR2-siRNA attenuates Ang II-induced expression of TLR2, TLR4 and MyD88. (A) mRNA expression of TLR2, TLR4 and MyD88. (B) Protein level of TLR2, TLR4 and MyD88. *P<0.05 vs. control group; #P<0.05 vs. Ang II + siRNA-NC (n=6). TLR, Toll-like receptor; si, small interfering; Ang II, Angiotensin II; MyD88, major myeloid differentiation response gene 88; NC, negative control.

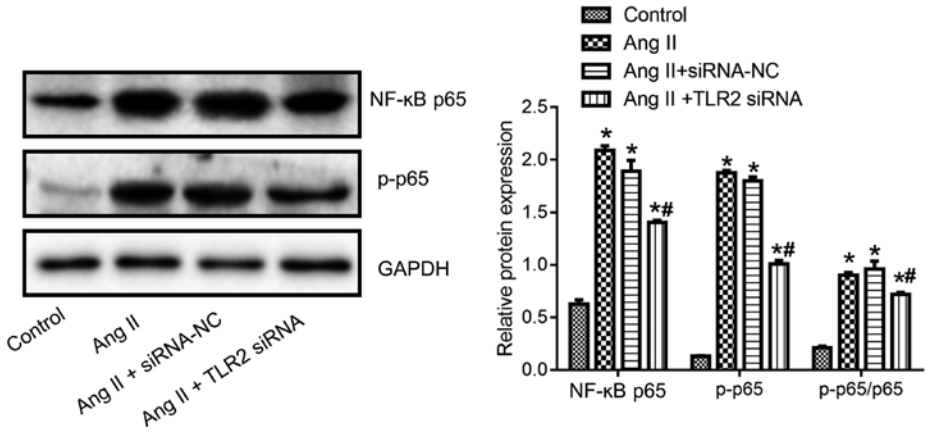

Figure 8. TLR2-siRNA attenuates Ang II-induced expression of NF-κB p65, p-p65 and p-p65/p65. *P<0.05 vs. control group; #P<0.05 vs. Ang II + siRNA-NC (n=6). TLR, Toll-like receptor; si, small interfering; Ang II, Angiotensin II; p-, phosphorylated; NC, negative control.

related to the occurrence and development of intracranial aneurysm (20). Ang II can cause vascular endothelial cell remodeling and induce vascular tissue dysfunction (21,22). Ang II serves an important role in myocardial remodeling by promoting the growth of cardiac fibroblasts and expression of extracellular matrix protein (23). It has been shown that Ang II stimulates the secretion of aldosterone in the adrenal cortex to regulate vasomotion, promotes the inflammatory response and oxidative stress of blood vessels and leads to the occurrence of vascular remodeling by acting on the type 1 receptor of angiotensin (24). Ang II stimulation can also promote the proliferation and migration of vascular smooth muscle (25). Thus, Ang II-stimulated BVSMCs were used to investigate the mechanisms underlying the proliferation and migration of vascular smooth muscle (25).

TLR2 and TLR4 are associated with the pathogenesis of vascular inflammation, atherosclerosis and diabetes (26-28). These receptors share a common downstream signaling pathway and serve a synergistic role in the activation of pre-inflammatory response (29,30). TLR2 and TLR4 are highly expressed in macrophages and endothelial cells in human atherosclerotic plaques, both of which promote the proliferation of vascular smooth muscle cells in animal atherosclerotic models (31,32). By knocking down the expression of TLR2, it was found that the effects of Ang II stimulation on human BVSMCs were blocked. These data suggested that TLR2 was involved in Ang II-induced cell remodeling.

As an inflammatory transcription factor, NF-κB is involved in many cell activities, including cell proliferation, stress response, immune response, apoptosis and inflammation (33,34). The activity of NF-κB in aneurysms is markedly increased (35,36), suggesting that NF-κB signaling pathway serves an important role in the occurrence and development of aneurysms. TLRs recognize the ligands and send signals to the intracellular domain through leucine rich repetitive sequences in the extracellular domain, and induce the corresponding signal conversion cascade reaction (37). Its signal transduction mechanism may include MyD88-dependent and independent mechanisms, which eventually lead to the activation of NF-κB (37). Using clinical samples, in the present study it was found that the expression levels of TLR2, TLR4, MyD88 and NF-κB p65 protein in patients with intracranial aneurysm were higher compared with normal control. These results also indicated that the TLR2/4/NF-κB signaling pathway served important roles in intracranial aneurysm.

The *in vitro* data of the present study demonstrated that the expression of TLR2, TLR4, MyD88, NF-κB p65 and p-p65 in BVSMCs stimulated by Ang II increased, but were downregulated by TLR2 silencing. Ang II can activate TLR4 and increase its expression (38). MyD88 can independently regulate cell activation and movement, and the TLR4/MyD88 signaling pathway is involved in Ang II-induced apoptosis (39). Ang II pretreatment results in upregulation of NF-κB and p-p65 expression (40). NF-κB is considered to be a typical pro-inflammatory molecule, regulating the transcription of a number of inflammatory cytokines (41). The results of the present study indicated that the TLR2/4-MyD88-NF-κB signaling pathway can be activated by Ang II treatment.

There remained some limitations to the present study. First, more clinical data ought to be collected to verify that the TLR2/4-MyD88-NF-κB signaling pathway is specific for the occurrence or development of intracranial aneurysm, but not subarachnoid hemorrhage. Subarachnoid hemorrhage can be caused by a ruptured aneurysm, arteriovenous malformation, or head injury (3,4). The present study did not distinguish subarachnoid hemorrhage patients from intracranial aneurysm patients. Future research should investigate the influence of subarachnoid hemorrhage caused by other factors on this signaling pathway. Second, Ang II treatment could affect the biological activities of BVSMCs, including the proliferation, apoptosis and migration. The TLR2/4-MyD88-NF-κB signaling pathway is an important pathway for inflammatory response (40). Whether inflammatory reaction takes part in the process deserves future study. Finally, 10% FBS was used in the cell migration assay. FBS may have also promoted the migration of the cells, which may cause bias or reduce the ability to observe the full effect of the knockdown of TLR2.

In conclusion, TLR2, TLR4, MyD88 and NF-κB p65 protein expression increased in patients with aneurysms, and the occurrence of aneurysms was related to the TLR2/4-MyD88-NF-κB signaling pathway. Interference of TLR2/4-MyD88-NF-κB signaling pathway will affect aneurysms.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ, YW, JF and ML performed the experiments and analyzed the data. XZ and ZJ designed the study, wrote the manuscript and confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consents were obtained from the participants. All experimental procedures were approved by the Ethics Committee of Nanchang University (approval no. NCU-20201213).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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