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Original article

Effect of notch1,2,3 genes silicing on NF-κB signaling pathway of macrophages in patients with atherosclerosis

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ABSTRACT

Background: Notch and NF-κB signaling pathways both play important roles in the regulation of atherosclerosis (AS). However, the mechanisms of notch and NF-κB signaling pathways on AS are still unclear. In this study, we aimed to investigate the effects of notch1,2,3 genes silicing by siRNA on notch and NF-κB signaling pathways of macrophages in patients with atherosclerosis (AS), so as to seek the treatment of AS from genetic perspective.

Methods: Peripheral blood mononuclears of 31 patients with AS were isolated by density gradient centrifugation and transformed by PMA to macrophages. Then macrophages were transfected with notch1-siRNA (notch1-siRNA group), notch2-siRNA (notch2-siRNA group), notch3-siRNA (notch3-siRNA group), negative control siRNA (NC group) and none (control group). RT-PCR and Western blot analysis were applied to assess the expression level of Delta-like-4 (DLL4), Jagged-1 (JAG1), IκBα and P52. Electrophoretic mobility shift assay (EMSA) was used to observe the NF-κB DNA binding activity. Subcellular distributions of NF-κB/P52 were detected through immunofluorescence. mRNA expression levels of TNF-α, IL-6 and IL-6 in macrophages were also determined with RT- PCR. The expression of 20S proteasome was detected by Western blot.

Results: After transfected with siRNA, there was no difference in the expression of DLL4, JAG1, IκBα and P52 between NC group and control group ($p > 0.05$). Compared with NC group and control group, the expression of DLL4, P52 and JAG1 in notch1-siRNA group, notch2-siRNA group and notch3-siRNA group was significantly downregulated ($p < 0.05$ or $p < 0.01$, respectively), whereas the expression of IκBα was significantly increased ($P < 0.05$ or $p < 0.01$, respectively), especially in notch1-siRNA group. The binding activity of NF-κB DNA was lower in notch1- siRNA group, notch2-siRNA group and notch3-siRNA group compared with NC group and control group ($p < 0.05$), especially in notch1-siRNA group. The fluorescence intensity of p52 was decreased significantly both in the nucleus and cytoplasm in notch1-siRNA group, notch2-siRNA group and notch3-siRNA group compared with NC group and control group ($p < 0.05$), which decreased more obviously in the nucleus, especially in notch1-siRNA group. The TNF-α, IL-1 and IL-6 expression of notch1-siRNA group, notch2-siRNA group and notch3-siRNA group was lower compared to NC group and control group ($p < 0.05$ or $p < 0.01$, respectively), also especially in notch1-siRNA group. 20S proteasome level was significantly lower in notch1-siRNA group, notch2-siRNA group and notch3-siRNA group than in NC group and control group ($p < 0.05$ or $p < 0.01$, respectively), especially in notch1-siRNA group.

Conclusions: There was a positive regulation between Notch and NF-κB signaling pathway in patients with AS. Notch1 may play a more important role than notch2 and notch 3 in the regulation of NF-κB signaling pathway in AS.

1. Introduction

Atherosclerosis (AS), which results in the gradual accumulation of atheromatous plaques in the wall of coronary, cerebral artery and aorta, is the most frequent cardiovascular disorder and the leading risk factor related with the cause of death throughout the

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world [1]. Inflammation plays an important role in the initiation and progression of AS [2]. Macrophages were the first inflammatory cells associated with AS, also the key players in all stages of AS [3]. The notch pathway is involved in cell proliferation, differentiation and apoptosis. Humans have four notch receptors (notch 1–4) and five ligands (Delta-like-1, 3, 4 and Jagged-1 and -2). Both receptors and ligands are located on the cell surface and regulate communication of adjacent cells [4]. Although a correlation between notch signaling and inflammation has been established, and notch signalling, by controlling the biology of endothelial cells, plays a major role in the first events leading to the formation of atherosclerotic plaques, inactivation of the notch pathway may mitigate AS [5,6], the exact mechanisms have yet not to be elucidated. In the inflammatory mechanism of initiating the occurrence of AS, NF-κB also plays one of the most important roles as multifunctional transcription regulators. Once activated, it exerts crucial effects on inflammatory reaction, immune reaction, cell proliferation, and apoptosis by regulating gene expressions of inflammatory cytokines and chemokines, such as IL-6 and TNF-α [7].

As discussed, notch and NF-κB signaling pathways both play important roles in the regulation of AS. However, the mechanisms of notch and NF-κB signaling pathways on AS are still unclear. It also remains unclear whether there is an interaction between notch and NF-κB signaling pathways in the process of AS. In the present study, we focused our inquiry to investigate the effects of notch1,2,3 genes silicing by siRNA on notch and NF-κB signaling pathways of macrophages in patients with AS, and the crosstalk between notch and NF-κB signaling pathways, so as to seek the treatment of AS from genetic perspective.

2. Materials and methods

2.1. Materials

Phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma-Aldrich, St. Louis (MO, U.S.A.). Notch1-siRNA, notch2-siRNA, notch3-siRNA and normal goat working serum were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Lipofectamin 2000, Trizol reagent and primers for DLL4, JAG1, GAPDH, IκBα, P52 were obtained from Invitrogen Company (California, USA). RT-PCR kit was from Thermo Fisher (Waltham, MA, USA). PVDF membrane was obtained from Millipore Corporation (Bedford MA, USA). Nuclear and cytoplasmic extraction reagents, primary antibody against IκBα, P52, ECL Western blotting Substrate and goat anti-rabbit IgG secondary antibody, EMSA detection kit, immunofluorecence kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Primary antibodies against JAG1, DLL4 and 20s proteasome were from Beijing Biosynthests Biotech. CO. LTD. (Beijing, China). P52 antibody was from Nanjing KeyGen BioTech company (Nanjing, China). UV-scanning was obtained from Fotodyne company (Washington, USA). 4′,6-diamidino-2-phenylindole (DAPI) was from Invitrogen company (California, USA), Syngene G:Box imaging System from Gene Company Ltd. (Hong Kong, SAR, China) and fluorescence microscope from Olympus company (Tokyo, Japan).

3. Methods

3.1. Induction to macrophages from peripheral blood mononuclear cells of patients with AS

20 ml of vein blood from 31 AS patients diagnosed by coronary arteriography at Taizhou People's Hospital in December 2014 was subjected respectively to density gradient centrifugation. Mononuclear cells were separated, washed and resuspended in PBS. The cells were then cultured in RPMI 1640 medium supplemented with FBS in 37 °C, 50 ml/l CO_2. After 3–5 passages, cells were seeded in 6-well plates with density of 0.5–1.0 × 10^6/ml, and stimulated with 100 ng/ml of PMA for 72 h, so by mononuclear cells differentiate into macrophages. Written informed consent was obtained from all patients, and the study protocol was approved by the Ethics Committee of Taizhou People's Hospital.

3.2. Small interfering RNA (siRNA) design and transfection into macrophages

Four pairs of siRNA were chosen for human notch1, notch2 and notch3 gene respectively by searching GenBank database and blasting. The sequences of notch1-siRNA, notch2-siRNA, notch3-siRNA and negative control were listed in Table 1. No homology was found for each pair of siRNA. For biomaterials selection, 5 μl of 20 μM siRNA was transferred to the 384-well V-bottom plate (Greiner Bio-one) containing 0–7 μl of dH2O with 2 μl of 0.8 M sucrose and diluted in Opti-MEM (Gibco, USA), then incubated for 5 min at room temperature. 5 μl of Lipofectamine 2000 was also diluted in Opti-MEM and incubated for 5 min at room temperature. The complexes of siRNA and transfection reagent were further incubated for 20 min at room temperature and added to the plates seeded with macrophages (2 × 105 cells/ml) for incubation 4 h. Then the complexes of siRNA and transfection reagent were removed by washing 3–5 times with PBS and the plates were incubated in CO_2 incubator for 24 h. Samples were taken for detecting the expression of Notch gene by real-time polymerase chain reaction (RT-PCR). The best silencing of siRNA with efficiency >80% was used for the experiment.

3.3. Detection of DLL4, JAG1, P52 and IκBα mRNA by RT-PCR

Total RNA was isolated with TRIzol reagent, and then reversely transcribed to cDNA, which was used for PCR amplification with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control according to the protocol. PCR condition was as follows: 3 min at 95 °C, followed by 45 cycles with denaturing

Table 1
Sequences of Notch1-, Notch2-, Notch3-siRNA and Negative control.

Gene	Primer sequence
Notch-1 siRNA-1	F(5-3): GCUCCCUCAACUUCAAUGAUU
	R(3-5): UUCGAGGGAGUUGAAGUUACU
Notch-1 siRNA-2	F(5-3): GCAGCGAGAACAUUGAUGATT
	R(3-5): TTCGUCGCUCUUGUAACUACU
Notch-1 siRNA-3	F(5-3): CAGGGAGCAUGUGUAACAUTT
	R(3-5): TTGUCCCUCGUACACAUUGUA
Notch-1 siRNA-4	F(5-3): CACCCAUGGUACCAAUCAUTT
	R(3-5): TTGUGGGUACCAUGGUUAGUA
Notch-2 siRNA-1	F(5-3): GCACCUGUGAGAGGAAUAUTT
	R(3-5): TTCGUGGACACUCUCCUUAUA
Notch-2 siRNA-2	F(5-3): GGCACAGGAUACUGCAAAUTT
	R(3-5): TTCCGUGUCCUAUGACGUUUA
Notch-2 siRNA-3	F(5-3): GGAGGUCUCAGUGGAUAUATT
	R(3-5): TTCCUCCAGAGUCACCUAUAU
Notch-2 siRNA-4	F(5-3): GGCAGCUGCUGUCAAUAAUTT
	R(3-5): TTCCGUCGACGACAGUUAUUA
Notch-3 siRNA-1	F(5-3): GGGCUUCAGUCUUCCUUUATT
	R(3-5): TTCCCGAAGUCAGAAGGAAAU
Notch-3 siRNA-2	F(5-3): GGCGUCAACACCUAUAACUTT
	R(3-5): TTCCGCAGUUGUGGAUAUUGA
Notch-3 siRNA-3	F(5-3): CUGGCUACAAUGGUGAUAATT
	R(3-5): TTGACCGAUGUUACCACUAUU
Notch-3 siRNA-4	F(5-3): CAGCCAUGCUGAUGUCAAUTT
	R(3-5): TTGUCGGUACGACUACAGUUA
NC	F(5-3): UUCUCCGAACGUGUCACGUTT
	R(3-5): TTAAGAGGCUUGCACAGUGCA

NC: negative control.