Journal of Bacteriology and Virology 2015. Vol. 45, No. 3 p.242 – 249
http://dx.doi.org/10.4167/jbv.2015.45.3.242

Original Article

Association between Polymorphisms in Toll-like Receptor 9 Gene and Outcomes after Ischemic Stroke

Jay Chol Choi[1], Keun-Hwa Lee[2*], Young Ree Kim[3] and Chang-Seok Ki[4]

[1]Department of Neurology, Jeju National University, Jeju; [2]Department of Microbiology and Immunology, Jeju National University, Jeju; [3]Department of Laboratory Medicine, Jeju National University, Jeju; [4]Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Several evidences suggested that Toll-like receptor 9 (*TLR9*) plays an important role in atherosclerosis and neuroprotection but the association between the *TLR9* and risk for stroke or outcomes after stroke has not been investigated. The aim of the present study was to investigate the association between *TLR9* polymorphisms and the risk for ischemic stroke using a case-control study design. We also explored the correlation between the polymorphisms and outcomes after stroke. We enrolled consecutive Korean stroke patients and controls without history of stroke. Four polymorphisms, namely c.-1486T>C, c.-1237C>T, c.1174A>G, and c.2848G>A were examined using polymerase chain reaction followed by direct sequencing. Initially we examined 193 stroke patients and the same number of healthy adults who had no history of stroke as controls. Due to deviation from Hardy-Weinberg equilibrium of initial controls, we performed genetic analysis of two polymorphisms (c.1174A>G and c.2848G>A) for additional 165 controls. The genotype frequency of four polymorphisms did not differ significantly between stroke patients and controls in unadjusted analysis. The variant allele (C) in c.-1486 locus was associated with significantly increased chance of favorable functional outcome at three month after stroke (OR 2.32, 95% CI 1.02~5.26, $p = 0.043$).

Key Words: Toll-like receptor, Polymorphism, Ischemic stroke, Outcomes

INTRODUCTION

Atherosclerosis in carotid artery or intracranial artery is one of the most important causes of ischemic stroke (1). For initiation and progression of atherosclerosis, inflammation and immune systems play an important role (2, 3). Both innate and adaptive immune systems are associated with atherosclerosis. Toll-like receptor (TLR), a pattern recognition receptor usually expressed on membranes of macrophage, is central to the innate immune system (4, 5). Genetic variations in genes encoding several TLRs affected the susceptibility of certain infectious disease and atherosclerosis (6).

TLR9 produces various pro-inflammatory cytokines and plays an important role in the cellular response to bacterial DNA (7). Recently several evidences suggested that *TLR9* plays an important role in atherosclerosis. The activation of

Received: June 19, 2015/ Revised: June 27, 2015/ Accepted: June 29, 2015
*Corresponding author: Keun Hwa Lee Ph.D. Department of Microbiology and Immunology, Jeju National University School of Medicine Jeju, 690-756, Korea.
Phone: +82-64-754-8111, Fax: +82-64-705-2687, e-mail: yomust7@jejunu.ac.kr
**This research was supported by the research grant from Jeju National University Hospital.
The biospecimens and data used in this study were provided by the Biobank of Jeju University Hospital, a member of Korea Biobank Network (No. A-01).

TLR9 facilitated the formation of foam cells *in vitro* (8) and *TLR9* expression was found in human carotid atherosclerotic plaque (9). In contrast, preconditioning with a TLR9 ligand induced neuroprotection against ischemic injury in mice stroke model, which was mediated by brain tumor necrosis factor (10, 11). However, the association between the *TLR9* polymorphisms and risk of stroke or the effect of *TLR9* polymorphism on stroke severity or clinical outcome has not been investigated yet. Therefore, the aims of the present study are to investigate whether the *TLR9* polymorphisms increase the risk of stroke using a case-control study design, and to examine the effects of the *TLR9* polymorphisms on stroke severity and outcomes among Korean stroke patients.

PATIENTS AND METHODS

Study participants

We enrolled consecutive Korean patients with acute ischemic stroke aged more than 20 years old, who had been admitted to Jeju National University Hospital within 7 days of symptom onset between Apr 2009 and May 2010. They were diagnosed as acute ischemic stroke through neurological examination, cranial computed tomography, or magnetic resonance imaging. The controls were healthy Korean adults who visited the hospital for a regular checkup and had no history of stroke. To verify the stroke free status, the interviewer asked each control if he or she had been advised as having had a stroke by a physician or had ever experienced hemiparesis, hemisensory loss, or speech difficulty. We received written informed consent from all participants and this study was approved by the Institutional Review Board of Jeju National University Hospital.

Clinical and laboratory assessment

Fasting venous blood was drawn for laboratory evaluation of hemoglobin, fasting blood glucose from all participants. We measured height, body weight, systolic and diastolic blood pressure from all participants. Body mass index was calculated by dividing the body weight (in kilograms) by the height (in meters) squared.

Hypertension was defined as a blood pressure of at least 140/90 mmHg or the use of an antihypertensive agent. Diabetes mellitus was defined as a fasting blood glucose level of at least 126 mg/dl or the use of antidiabetic medication. Smoking status was determined by self-reporting as a smoker (current or ex-smoker) or nonsmoker. Ischemic stroke was classified as large-artery atherosclerosis (LAA), small-vessel occlusion (SVO), cardioembolism (CE), ischemic stroke of undetermined etiology (UDE), or ischemic stroke of other determined etiology (ODE) according to the Trial of Org 10172 in Acute Stroke Treatment criteria (12). The severity of stroke was measured using initial National Institutes of Health Stroke Scale (NIHSS) score on the day of admission (13). Functional outcome of index stroke was measured at three months after onset using modified Rankin score (mRS) (14). We dichotomized clinical outcome as favorable (mRS 0 to 1) versus poor (mRS 2 to 6).

Genetic analysis

In this study, we examined four polymorphisms, namely c.-1486T>C, c.1237C>T, c.1174A>G, c.2848G>A, which showed higher minor allele frequencies among *TLR9* polymorphisms (15). DNA was extracted from peripheral blood from the participants using the bead beater-phenol extraction method (16). The exact sequences of the primers used for genotyping of the four polymorphisms were provided as Table 1. Polymerase chain reaction (PCR) for c.-1486T>C, c.-1237C>T, c.1174A>G, c.2848G>A were performed respectively. The PCR parameters were 5 min at 95℃, followed by 40 cycles of 45 sec at 94℃, 45 sec at 60℃, 60 sec at 72℃ with termination using a final extension step at 72℃ for 10 min. The PCR products were electrophoresed on a 1.2% agarose gel and purified using a QIAEX II gel extraction kit (Qiagen Inc, Hilden, Germany) according to the manufacturer's instructions and sequenced using a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, UK) respectively. Nucleotide sequences were analysed using BioEdit software (version 5.0.9.1; T. A. Hall Software, Ibis Biosciences, Carlsbad, CA), CHROMAS version 2.33 and BLAST (Basic Local Alignment Search Tool).

Table 1. The sequences of the primers used for the genotypes of four polymorphisms in this study

Locus	Direction	Sequence
c.-1486T>C	Forward	5'-TCATTCAGCCTTCACTCAGAAA-3'
	Reverse	5'-ACCTCCCACCCCAGATCT-3'
c.-1237C>T	Forward	5'-TAAGAAGGCTGGATGGCCCT-3'
	Reverse	5'-GGGACCTGCCACCCG-3'
c.1174A>G	Forward	5'-AATTCTGAGTCCAAGACTGGGTCT-3'
	Reverse	5'-GCTGGAGCTCACAGGGTAGG-3'
c.2848C>T	Forward	5'-AGGCTGAGGTGGCGCA-3'
	Reverse	5'-CGGAGATGTTTGCCCAGCT-3'

Statistical analysis

For statistical analysis, we compared the baseline characteristics between the stroke patients and controls using chi-square test for categorical variables and Student *t*-test for continuous variables. To assess deviation from Hardy-Weinberg equilibrium (HWE) and to compare the genotype and allele frequency between controls and stroke patients, chi-square test was used for the analysis (17). After unadjusted analysis, multivariable logistic regression analysis was used to estimate independent association between polymorphism and risk for stroke with adjustments for age, sex, laboratory findings and vascular risk factors. Since the atherothrombotic type of ischemic stroke was expected to show significant associations with *TLR9* polymorphisms based on previous research, we planned to repeat the analysis for patients with atherothrombotic subtypes of ischemic stroke, namely LAA or SVO to investigate the association between *TLR9* polymorphisms and risk of atherothrombotic stroke for subgroup analysis. To explore the associations between *TLR9* polymorphisms and stroke severity, we used analysis of covariance with the baseline NIHSS score as a dependent variable to adjust for other covariates. Multivariable logistic regression analysis was used to assess the association between *TLR9* polymorphisms and stroke outcomes at three month in addition to unadjusted analysis. Stata 12 (StataCorp. TX, USA) for Windows was used for all statistical analyses.

RESULTS

Characteristics of participants

During enrollment period, 199 stroke patients were admitted. Among them, six patients refused the participation. In addition, 193 healthy adults who had no history of stroke were enrolled as controls. The mean age of 386 participants were 66±12 years, and 60% were men. Compared with controls, stroke patients were more likely to be older, to have hypertension, diabetes mellitus, and smoking habit, and to show elevated fasting blood glucose, systolic and diastolic blood pressure (Table 2).

Of 193 patients with ischemic stroke, almost 90% (173 patients) were diagnosed with cerebral infarction; the remaining 10% (20 patients) were diagnosed with transient ischemic attack. LAA was the most common subtype of ischemic stroke (51 patients, 26.4%), followed by SVO (49 patients, 25.4%), UDE (48 patients, 24.9%), CE (19 patients, 9.8%), and ODE (6 patients, 3.1%). Brain MRI was performed in 181 patients (93.8%). MRA was performed in 169 patients (87.6%) and conventional catheter cerebral angiography was done in 21 patients (10.9%). The median with interquartile range (IQR) of the initial NIHSS was 3 (1~6). Clinical outcomes were available for 181 patients; favorable clinical outcomes (mRS 0-1) were found in 122 patients (67.4%) at three months after the index stroke.

Table 2. Baseline characteristics of 193 stroke patients and 193 controls

	Control (*n*=*193*)	Stroke (*n*=*193*)	*p*
Demographic characteristics			
Age, years	67±12	65±12	<0.001
Sex, men	116 (60.1)	116 (60.1)	1.000
BMI, kg/m^2	24.8±3.2	24.1±3.3	0.068
Vascular risk factors			
Hypertension	67 (34.7)	120 (62.2)	<0.001
Diabetes mellitus	30 (15.5)	57 (29.5)	0.001
Smoking	58 (30.1)	84 (43.5)	0.006
Laboratory findings			
Hemoglobin	13.1±2.1	13.7±2.1	0.009
Fasting blood glucose, mg/dl	110±46	131±53	<0.001
Systolic blood pressure, mmHg	131±17	156±26	<0.001
Diastolic blood pressure, mmHg	79±12	88±14	<0.001

BMI, body mass index. Data are mean ± SD values or *n* (%). Analyzed by *t*-test, Wilcoxon signed rank test, or chi-square test according to the type of variables

Results of genetic analysis

For c.-1237C>T polymorphism, the frequency of minor allele was only 2% and no homozygote was found for C allele. The genotype frequency of two promotor region polymorphisms (c.-1486T>C and c-1237C>T) did not significantly differ between stroke patients and controls (chi-square test, $p = 0.126$ and $p = 0.787$, respectively). The risk for stroke did not differ significantly between stroke patients and controls by genotypes, and it remained nonsignificant after adjustments for age, sex, BMI, history of hypertension, diabetes mellitus, smoking, hemoglobin, and fasting blood glucose (Table 3).

Unexpectedly, the distributions of c.1174A>G and c.2848C>T in control subjects showed significant deviation from the estimated frequency of HWE (chi-square test, $p = 0.013$ and $p = 0.059$, respectively) possibly due to random bias. Therefore, we performed genetic analysis of two polymorphisms for additional 165 controls without history of stroke. After addition of 165 controls, the deviation from the distribution of HWE disappeared (chi-square test, $p = 0.129$ and $p = 0.204$, respectively). The frequency of geno-

Table 3. Genotype distribution of the c.-1486T>C and c.-1237T>C promotor polymorphisms between stroke patients and controls and the unadjusted and adjusted risk of stroke by the genotypes

Locus	Genotype	Control (%) (*n*=*193*)	Stroke (%) (*n*=*193*)	Odd ratio (95% CI)	*p*	Adjusted OR* (95% CI)	*p*
c.-1486T>C	TT	62 (32.1)	76 (39.4)	Reference	−	Reference	−
	TC	107 (55.4)	87 (45.1)	0.66 (0.42~1.05)	0.066	0.85 (0.46~1.55)	0.594
	CC	24 (12.4)	30 (15.5)	1.02 (0.52~2.02)	0.952	1.28 (0.85~1.92)	0.242
	TC+CC	131 (67.9)	117 (60.6)	0.73 (0.47~1.13)	0.137	1.01 (0.58~1.76)	0.966
	Minor allele frequency	77 (40.0)	73 (38.0)	0.92 (0.60~1.41)	0.676		
c.-1237T>C	TT	187 (96.9)	185 (95.9)	Reference	−	Reference	−
	TC	6 (3.1)	8 (4.1)	1.35 (0.40~4.81)	0.586	2.19 (0.53~9.09)	0.279
	CC	0	0	NA	NA	NA	NA
	TC+CC	6	8	1.35 (0.40~4.81)	0.586	2.19 (0.53~9.09)	0.279
	Minor allele frequency	4 (2.0)	4 (2.0)	1.00 (0.18~5.45)	1.000		

*Adjusted for age, sex, BMI, history of hypertension, diabetes mellitus, smoking, hemoglobin, and fasting blood glucose

Table 4. Genotype distribution of the c.1174A>G and c.2848C>T polymorphisms between stroke patients and controls and risk of stroke by the genotype

Locus	Genotype	Control (*n=358*)	Stroke (*n=193*)	Odd ratio (95% CI)	*p*	Adjusted OR* (95% CI)	*p*
c.1174A>G	AA	118 (33.0)	71 (36.8)	Reference	−	Reference	−
	AG	187 (52.2)	89 (46.1)	0.79 (0.53~1.19)	0.236	1.00 (0.60~1.67)	0.999
	GG	53 (14.8)	33 (17.1)	1.03 (0.59~1.80)	0.898	1.18 (0.84~1.66)	0.339
	AG+GG	240 (67.0)	122 (63.2)	0.84 (0.58~1.24)	0.367	1.08 (0.67~1.73)	0.761
	Minor allele frequency	147 (40.0)	77 (40.0)	0.95 (0.66~1.38)	0.790		
c.2848C>T	CC	115 (32.1)	75 (38.9)	Reference	−	Reference	−
	CT	186 (52.0)	86 (44.6)	0.71 (0.47~1.06)	0.081	1.04 (0.62~1.73)	0.881
	TT	57 (15.9)	32 (16.6)	0.86 (0.49~1.49)	0.573	1.13 (0.81~1.57)	0.474
	CT+TT	243 (67.9)	118 (61.1)	0.74 (0.51~1.09)	0.113	1.09 (0.68~1.74)	0.730
	Minor allele frequency	150 (42.0)	75 (39.0)	0.74 (0.51~1.09)	0.113		

*Adjusted for age, sex, BMI, history of hypertension, diabetes mellitus, smoking, hemoglobin, and fasting blood glucose

Table 5. Baseline NIHSS score by *TLR* polymorphisms

Polymorphism	Unadjusted analysis			Adjusted analysis*		
	Wild	Variant	*p*	Wild	Variant	*p*
c.-1486T>C	5.1 (3.7~6.5)	4.4 (3.5~5.3)	0.283	4.3 (2.0~6.5)	3.4 (1.3~5.6)	0.269
c.-1237T>C	4.6 (3.8~5.4)	6.4 (1.8~11.0)	0.236	3.9 (1.8~6.0)	5.5 (0.7~10.2)	0.441
c.1174A>G	4.8 (3.9~5.6)	4.2 (2.5~6.0)	0.390	4.0 (1.9~6.1)	2.9 (0.3~5.5)	0.281
c.2848C>T	5.1 (3.7~6.5)	4.4 (3.5~5.3)	0.634	4.1 (1.8~6.3)	3.5 (1.3~5.71)	0.498

Values are mean (95% CI) or adjusted mean (95% CI) *Adjusted for age, sex, onset to arrival time, hypertension, diabetes mellitus, hyperlipidemia, atrial fibrillation, smoking, fasting blood glucose and TOAST classification. NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin score.

types did not differ significantly between 193 stroke patients and 358 controls for those two polymorphisms (chi-square test, $p = 0.388$ and $p = 0.215$, respectively). The risk for stroke did not differ significantly between stroke patients and controls by genotypes, and it remained nonsignificant after adjustments for age, sex, BMI, history of hypertension, diabetes mellitus, smoking, hemoglobin, and fasting blood glucose (Table 4). Similarly, for subgroup analysis with 100 atherothrombotic stroke patients and controls, the risk for stroke did not differ significantly by genotypes of four *TLR9* polymorphisms both in unadjusted and in adjusted analyses.

Associations of *TLR9* polymorphisms on stroke severity and outcome

Initial stroke severity measured by NIHSS score did not differ significantly between the patients with wild type and the patients with variant allele for all polymorphisms both in unadjusted analysis and adjusted analysis (Table 5). The proportion of favorable outcome at three month (mRS 0 to

Table 6. Unadjusted and adjusted odd ratios for favorable clinical outcomes at three month by TLR genotypes

Polymorphism	Unadjusted OR (95% CI)	*p*	Adjusted OR (95% CI)	*p*
c.-1486T>C	1.60 (0.81~3.15)	0.142	2.32 (1.02~5.26)	0.043
c.-1237T>C	0.47 (0.06~3.64)	0.355	1.24 (0.11~13.38)	0.861
c.1174A>G	1.23 (0.48~3.45)	0.653	1.11 (0.36~3.38)	0.859
c.2848C>T	1.17 (0.59~2.31)	0.620	1.74 (0.77~3.90)	0.180

Adjusted for age, sex, onset to arrival time, hypertension, diabetes mellitus, hyperlipidemia, smoking, atrial fibrillation, fasting blood glucose, TOAST classification, and initial NIHSS scores.

1) did not differ significantly between wild and variant allele for all polymorphisms in unadjusted analysis. After adjustments for age, sex, onset to arrival time, hypertension, diabetes mellitus, hyperlipidemia, atrial fibrillation, smoking, fasting blood glucose and TOAST classification, and initial NIHSS score, the patients having the variant allele (C) in -1486 locus was associated with significantly increased chance of favorable functional outcome at three month (odd ratio, 2.32; 95% confidence interval, 1.02~5.26; $p = 0.043$) (Table 6).

DISCUSSIONS

The frequencies of genotypes of the four *TLR9* polymorphisms in this study were similar to those of other reports from Asian countries including China, Japan, and Korea (18~20). The frequency of variant allele in c.-1237T>C was only 2% and such rarity was also reported in the study from Japan (20). Since the significant deviation from HWE was found in c.1174A>G and c.2848C>T among control subjects, this led the analysis of additional 165 adults without history of stroke.

In this study, we could not find any association between the four *TLR9* polymorphisms and risk of stroke. The results could be explained by 1) since ischemic strokes have often multiple, heterogeneous mechanisms, the effect of polymorphisms on the risk of stroke might be different according to the mechanisms of ischemic stroke. Although we examined the frequencies of genotypes according to subtype of ischemic stroke and found no significant difference among them, the analysis was not adequately powered due to small sample size when divided into four subtypes of ischemic stroke; 2) although the four polymorphisms were known to show differences of functional activities of *TLR9* gene, those difference might not be sufficient to cause phenotypic change leading to stroke (21~23).

Among four TLR9 polymorphisms, we found that the patients having the variant allele (C) in c.-1486 locus was associated with significantly increased chance of favorable functional outcome at three month after adjustment for other clinical and laboratory findings. In patients with stroke, TLR2 and TLR4 expression was significantly higher in patients with poor clinical outcome and correlated well with the level of inflammatory makers including interleukin 1β, tumor necrosis factor α, and interleukin 6 (24, 25). In contrast, animal research showed preconditioning with TLR9 ligand CpG oligodeoxynucleotide induced neuroprotection against ischemic injury through cerebral tumor necrosis factor (10, 11). Because gene expression and subsequent inflammatory response could vary with TLR9 genotype in human, our findings may suggest the different level of ischemic preconditioning by TLR9 genotype which led to significant differences in favorable functional outcomes in stroke patients (26). Therefore, the exact role of different TLR9 genotypes on gene expression and resultant neuroprotection in human stroke should be investigated further in large scale clinical study because the ligand can be developed as a neuroprotective agent in human stroke prior to initiation of standard thrombolytic therapy.

This study is subject to several limitations. We had rather

small sample size that led to initial deviation from HWE. We had to perform genetic testing in additional 165 adults without history of stroke. The small sample size could also affect the results of adjusted analysis for the associations between TLR9 genotype and stroke severity or outcomes because those results had wide confidence intervals. Moreover, we did not have any laboratory information on the level of gene expression or biochemical markers for inflammatory response after stroke that could support functional difference by TLR9 genotypes. Future studies should validate our finding using large sample size and tests for gene expression and level of inflammation.

In conclusion, we could not find significant association between the four *TLR9* polymorphisms and risk of stroke in this study. However, the variant allele in c.-1486 locus was associated with significantly improved functional outcomes after stroke, which warrants further exploration.

REFERENCES

1) Petty GW, Brown RD Jr, Whisnant JP, Sicks JD, O'Fallon WM, Wiebers DO. Ischemic stroke subtypes: a population-based study of incidence and risk factors. Stroke 1999;30:2513-6.
2) Libby P. Inflammation in atherosclerosis. Nature 2002; 420:868-74.
3) Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.
4) Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675-80.
5) Frantz S, Ertl G, Bauersachs J. Mechanisms of disease: Toll-like receptors in cardiovascular disease. Nat Clin Pract Cardiovasc Med 2007;4:444-54.
6) Schröder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis 2005;5:156-64.
7) Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, *et al*. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740-5.
8) Sorrentino R, Morello S, Chen S, Bonavita E, Pinto A. The activation of liver X receptors inhibits toll-like receptor-9-induced foam cell formation. J Cell Physiol 2010;223:158-67.
9) Niessner A, Sato K, Chaikof EL, Colmegna I, Goronzy JJ, Weyand CM. Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha. Circulation 2006;114:2482-9.
10) Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, *et al*. Toll-like receptor 9: a new target of ischemic preconditioning in the brain. J Cereb Blood Flow Metab 2008;28:1040-7.
11) Packard AE, Leung PY, Vartanian KB, Stevens SL, Bahjat FR, Stenzel-Poore MP. TLR9 bone marrow chimeric mice define a role for cerebral TNF in neuroprotection induced by CpG preconditioning. J Cereb Blood Flow Metab 2012;32:2193-200.
12) Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, *et al*. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke 1993;24:35-41.
13) Adams HP Jr, Davis PH, Leira EC, Chang KC, Bendixen BH, Clarke WR, *et al*. Baseline NIH Stroke Scale score strongly predicts outcome after stroke: A report of the Trial of Org 10172 in Acute Stroke Treatment (TOAST). Neurology 1999;53:126-31.
14) Wolfe CD, Taub NA, Woodrow EJ, Burney PG. Assessment of scales of disability and handicap for stroke patients. Stroke 1991;22:1242-4.
15) Lazarus R, Klimecki WT, Raby BA, Vercelli D, Palmer LJ, Kwiatkowski DJ, *et al*. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 2003;81: 85-91.
16) Choi JC, Lee KH, Song SK, Lee JS, Kang SY, Kang JH. Screening for NOTCH3 gene mutations among 151 consecutive Korean patients with acute ischemic stroke. J Stroke Cerebrovasc Dis 2013;22:608-14.
17) Rodriguez S, Gaunt TR, Day IN. Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. Am J Epidemiol 2009; 169:505-14.

18) Hur JW, Shin HD, Park BL, Kim LH, Kim SY, Bae SC. Association study of Toll-like receptor 9 gene polymorphism in Korean patients with systemic lupus erythematosus. Tissue Antigens 2005;65:266-70.
19) Liu F, Lu W, Qian Q, Qi W, Hu J, Feng B. Frequency of TLR 2, 4, and 9 gene polymorphisms in Chinese population and their susceptibility to type 2 diabetes and coronary artery disease. J Biomed Biotechnol 2012; 2012:373945.
20) Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, *et al*. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007;66:905-9.
21) Hamann L, Glaeser C, Hamprecht A, Gross M, Gomma A, Schumann RR. Toll-like receptor (TLR)-9 promotor polymorphisms and atherosclerosis. Clin Chim Acta 2006;364:303-7.
22) Lange NE, Zhou X, Lasky-Su J, Himes BE, Lazarus R, Soto-Quirós M, *et al*. Comprehensive genetic assessment of a functional TLR9 promoter polymorphism: no replicable association with asthma or asthma-related phenotypes. BMC Med Genet 2011;12:26.
23) Roszak A, Lianeri M, Sowińska A, Jagodziński PP. Involvement of Toll-like Receptor 9 polymorphism in cervical cancer development. Mol Biol Rep 2012;39: 8425-30.
24) Brea D, Blanco M, Ramos-Cabrer P, Moldes O, Arias S, Pérez-Mato M, *et al*. Toll-like receptors 2 and 4 in ischemic stroke: outcome and therapeutic values. J Cereb Blood Flow Metab 2011;31:1424-31.
25) Wang Y, Ge P, Zhu Y. TLR2 and TLR4 in the brain injury caused by cerebral ischemia and reperfusion. Mediators Inflamm 2013;2013:124614.
26) Kikuchi K, Lian ZX, Kimura Y, Selmi C, Yang GX, Gordon SC, *et al*. Genetic polymorphisms of toll-like receptor 9 influence the immune response to CpG and contribute to hyper-IgM in primary biliary cirrhosis. J Autoimmun 2005;24:347-52.