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Single nucleotide polymorphism of *CD40* in the 5′-untranslated region is associated with ischemic stroke

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ABSTRACT

Objectives: Ischemic stroke is influenced by both environmental and genetic factors. The CD40/CD40L system is related to proinflammatory and prothrombogenic responses, which are involved in the pathophysiology of ischemic stroke. The aim of this study was to evaluate association between the CD40 -1C/T single nucleotide polymorphism (SNP) and ischemic stroke in a Chinese population.
Methods: We conducted a case–control study including 286 ischemic stroke patients and 336 controls. CD40 -1C/T SNP was genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing methods, and evaluated its relevance to ischemic stroke susceptibility.
Results: Significantly increased ischemic stroke risk was found to be associated with the T allele of *CD40* -1C/T (OR = 1.273, 95% CI = 1.016–1.594). The frequencies of CT and TT/CT genotypes of *CD40* -1C/T in ischemic stroke patients were significantly higher than those of controls, respectively (for CT: OR = 2.350, 95% CI = 1.601–3.449; for TT/CT: OR = 2.148, 95% CI = 1.479–3.119). And, similar results were obtained after adjusting non-matched variables. We found that the frequency of carried T genotypes (TT and TT/CT) was significantly increased in patients with history of stroke compared with patients without (for TT: OR = 6.538, 95%CI = 1.655–25.833; for TT/CT: OR = 3.469, 95%CI = 1.031–11.670), respectively.
Conclusions: The findings suggested that the *CD40* -1C/T polymorphism might contribute to the susceptibility to ischemic stroke in the Chinese population, and might be associated with history of previous stroke.

1. Introduction

Stroke, a major cause of death and long term disability, is a heavy economic and health burden for patients and societies in the world (Ma et al., 2010; Wolf, 1998). It is characterized by a high mortality rate in the acute phase, high disability and handicap, high recurrence, and low complete rehabilitation rate (Rosenberg and Popelka, 2000; Sturm et al., 2004; Wolfe et al., 2000). Ischemic stroke is the most common type of stroke(over 80% of all strokes)(Rosamond et al., 2008), which is a late-onset and complex polygenic disease, consisting of a group of heterogeneous disorders with multiple genetic and environmental risk factors (Dominiczak and McBride, 2003; Sacco et al., 1997). Many susceptibility genes associated with ischemic stroke have been identified. However, these susceptibility genes explained only a small fraction of the inherited risk of ischemic stroke; and the remainders were attributable to covariates such as lipids, diabetes, blood pressure, and smoking (Bersano et al., 2008; Dominiczak and McBride, 2003; Duggirala et al., 1996). Better knowledge of the genetic background and susceptibility genes would probably help us focus on early diagnosis and treatment, and improve ischemic stroke outcome in affected patients.

CD40 is a cell surface trimeric transmembrane glycoprotein from a tumor necrosis factor receptor (TNFR) family member, which is expressed on the surface of immune cells, including B cells, monocytes, and dendritic cells, as well as non-immune cells such as endothelial cells, epithelial cells, mesenchymal cells, platelets and malignant tumor cells (Chen et al., 2006). CD40 was located on 20q12–13.2, and determined T cell responses to antigen presentation and B cells immunoglobulin isotype switching (Chen et al., 2006). Recently, some studies have demonstrated that CD40/CD40 ligand (CD40L) was elevated on monocytes or in serum of patients after transient ischemic attack or

Abbreviations: SNP, single nucleotide polymorphism; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; TNFR, tumor necrosis factor receptor; CD40L, CD40 ligand; CNS, central nervous system; 5′-UTR, 5′-untranslated region; ICD-9-CM, International Classification of Diseases, 9th Revision, Clinical Modification; CT, computed tomography; MRI, magnetic resonance imaging; TOAST, Trial of Org10172 in Acute Stroke Treatment; LAA, large artery atherosclerosis; CE, cardioembolism; SVO, small vessel occlusion; NIHSS, National Institutes of Health Stroke Scale; OR, odds ratio; CI, confidence intervals; HWE, Hardy–Weinberg equilibrium; CHD, coronary heart disease; LD, linkage disequilibrium; sCD40, soluble CD40.

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stroke, and involved in the pathophysiology of ischemic stroke which was related to inflammatory response, thrombogenic components and proatherogenic milieu (Garlichs et al., 2003; Grau and Lichy, 2003; Ishikawa et al., 2005). CD40 mRNA and protein were also expressed on neuronal cells, and played a role in neuronal development, maintenance and protection both *in vitro* and *in vivo* (Tan et al., 2002). Besides, CD40 was detected on vascular endothelial cells, smooth muscle cells, astrocytes and microglia in the central nervous system (CNS) (Chen et al., 2006; Vowinkel et al., 2006). Interaction between CD40 on microglia and CD40L presented by infiltrating T lymphocytes and other resident CNS cells triggered a series of intracellular signaling events that promoted the production of a wide array of cytokines, chemokines and neurotoxins (Chen et al., 2006). These data demonstrated that CD40 might play an important role in ischemic stroke, and was likely a positional candidate gene for its risk.

Single nucleotide polymorphisms (SNPs) are the most frequent type of variation in the human genome (Wang et al., 1998). There are about 45 SNPs for the CD40 gene in the Chinese Han population (http://genome.ucsc.edu/cgi-bin/hgTracks). CD40 SNPs associated with infection- and autoimmunity-associated diseases were widely studied, *e.g.* acute coronary syndrome, Graves' disease (Burdon et al., 2006; Kurylowicz et al., 2005; Tian et al., 2010). However, only one known SNP affected CD40 protein levels, which was located at the -1 position in the 5′-untranslated region (5′-UTR) nearby the start codon, and coincided with the Kozak consensus sequence(CD40 -1C/T; refSNP ID: rs1883832) (Jacobson et al., 2005; Kozak, 1987; Tomer et al., 2002). Besides the CD40 -1C/T polymorphism which associated with acute coronary syndrome, coronary artery calcification was identified (Burdon et al., 2006; Tian et al., 2010), which might play an important role in the development of ischemic stroke. To our best knowledge, only del Rio-Espinola et al. reported that the CD40 -1C/T polymorphism might be associated with reocclusion risk after successful fibrinolytic therapy during the acute phase of ischemic stroke in Spanish (del Rio-Espinola et al., 2010). However, only little information was available on the association between the CD40 -1C/T polymorphism and genetic susceptibility to ischemic stroke. We hypothesized that the CD40 -1C/T polymorphism was a high-risk gene which might affect CD40 protein expression in ischemic stroke, and help refine the ischemic stroke risk profile. We tested this hypothesis by investigating the relationship between the CD40 -1C/T polymorphism and ischemic stroke risk, as well as the association between the SNP and risk factors of ischemic stroke through a case–control study derived from a general Chinese population.

2. Materials and methods

2.1. Study population

Participants were enrolled from the Department of Neurology, Affiliated Hospital of North-Sichuan Medical College. The study was performed with the approval of the hospital ethics committee, and written informed consents were obtained from all the subjects. The pilot study population consisted of 286 patients who suffered from ischemic stroke as the case group (174 men and 112 women, mean age: 66.08 ± 10.87 years) from July 2009 to July 2010, and 336 healthy volunteers as the control group (183 men and 153 women, mean age: 67.51 ± 10.39 years) during the same period. All the cases and controls selected from the same Chinese Han population were unrelated by self-description.

In our study, all the participants were adult men and women over the age of 35 years. Ischemic stroke diagnosis was made according to the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) code 433, 434, and 436 (Goldstein, 1998), and all cases were ascertained in accordance with World Health Organization criteria by two neurologists on the basis of history, physical examination, B-mode carotid ultrasonography, electrocardiography, and cranial computed tomography (CT) or magnetic resonance imaging (MRI). Covariates of age, gender, obesity, hypertension, diabetes mellitus, coronary heart disease, hyperlipidemia, previous stroke, and smoking status were carefully recorded. The ischemic stroke classification was according to the Trial of Org10172 in Acute Stroke Treatment (TOAST) criteria (Adams et al., 1993). TOAST subtypes were as follows: large artery atherosclerosis (LAA, n = 36); cardioembolism (CE, n = 22); small vessel occlusion (SVO, n = 188); other etiology (n = 40) including specific etiology (2 cases) and undetermined etiology (38 cases). Stroke severity on admission was assessed using the National Institutes of Health Stroke Scale (NIHSS). Individuals diagnosed with hemorrhagic stroke, transient ischemic attacks, sinus thrombosis, traumatic brain injuries, tumors, infective diseases, and spontaneous subarachnoid hemorrhages were excluded.

In the control group, individuals without clinical or radiological evidence of cerebrovascular diseases or arterial vascular diseases were recruited. Individuals were excluded if they had a history of stroke and hereditary diseases, tumors, or infective diseases in the past 4 weeks before the study. All the controls were similar to ischemic stroke patients in age, gender, and smoker status.

2.2. Genotyping

Genomic DNA of each individual was extracted from 200 μl EDTA-Na_2 anticoagulated peripheral blood samples according to the manufacturer's instructions of a commercial DNA isolation kit from Bioteke (Peking, China), and its concentration was determined using a NanoDrop® ND-1000 Spectrophotometer from Thermo Fisher Scientific Inc. (Waltham, USA).

Genotyping (*CD40* -1C/T) was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primer sequences, reaction conditions, and restriction enzymes used were described previously (Liu et al., 2009). In brief, the PCR was carried out in a total volume of 25 μl, containing 100 ng genomic DNA, 2.5 μl of 10× buffer, 0.5 μmol/l of primers, 0.15 mmol/l of dNTP, 1.5 mmol/L of $MgCl_{2,\ and}$ 1 U of *Taq* DNA polymerase (Tiangen Biotech, Peking, China). PCR was started with an initial denaturation step for 5 mins at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 65 °C, and extension for 30s at 72 °C, followed by a final extension step for 10 mins at 72 °C in a thermal-cycler (MyCycler™ Thermal Cycler; BIO-RAD, USA). The PCR products were digested by 2 U of *sty*I (New England BioLabs, Ltd) at 37 °C for 4 h, in which CD40 -1C/T allele C yielded four fragments including 85 bp, 11 bp, 74 bp and 133 bp, allele T yielded three fragments including 85 bp, 11 bp, and 207 bp. The digestion products were analyzed directly by a 6% non-denaturing polyacrylamide gel, stained with 1.0 g/L of argent nitrate as well (Fig. 1). To confirm the accuracy of the method used, the genotypes were confirmed by the DNA sequencing analysis, and at the same time, about 10% of the samples were randomly selected to perform the repeated assays and the results were 100% concordant.

2.3. Statistical analysis

Genotype and allele frequency of *CD40* -1C/T were compared between ischemic stroke patients and controls using the chi-square test and Fisher's exact test when appropriate, and odds ratio (OR) and 95% confidence intervals (CIs) were calculated to evaluate the effects of any difference between allelic and genotype distribution. Demographic and vascular risk factors between groups were compared by the chi-square test for categorical variables, and by a parametric (Student's *t*-test) or nonparametric test (Wilcoxon rank sum test) for differences in continuous variables. The Hardy–Weinberg equilibrium (HWE) was evaluated with a goodness of fit for the chi-square test with one degree of freedom to compare the observed genotype frequencies among the subjects with the expected genotype frequency. A multivariate logistic regression analysis was carried out to find the

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