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The research on association of copy number variation in chromosome 9p21 region with atherothrombotic stroke in the Han Chinese population

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ABSTRACT

Background/purpose: The copy number variants (CNVs) contain more genetic information compared with SNPs. The aim of this study was to elucidate whether the CNVs in Chromosome 9p21 region are associated with increased risk of Atherothrombotic stroke (ATS) in a Han Chinese population.
Methods: A case-controlled association study was conducted in which only patients with ATS were enrolled. The CNVs were detected by the method of multiplex competitive amplification. The differences in distribution of CNVs between cases and controls were analyzed using univariate and multivariate logistic regression analysis. Subgroup analyses were also carried out to determine whether the effect of the CNVs was specific to age and gender among the subjects.
Results: A total of 274 ATS patients and 282 health controls were included in the present study. 4 genes (ANRIL, CDKN2A, CDKN2B, and MTAP) including eight gene fragments in all were analyzed for CNV. The results showed that the copied number of most CNV in the 4 genes is two. There was no significant difference of CNV frequency between groups.
Conclusions: The obtained data suggested a negative association between CNV of the four genes and ATS. It is necessary to perform sequencing analyses across the entire 9p21 region for detecting rare or uncommon CNV.

1. Introduction

Stroke is the second most common cause of death [1] and the third most common cause of disability-adjusted life-years (DALYs) worldwide [2]. In mainland China, it is estimated that about 1.5–2.0 million new cases occur each year [3]. The cerebrovascular diseases were reportedly the leading cause of death in 2008 [4]. Most strokes are ischemic in origin, and up to 67.3–80.5% of which are attributed to ischemic stroke (IS) in developed countries and approximately 43.7–78.9% in China [3]. The studies suggested genetic influences might contribute to a predisposition to IS [5]. The elucidation of genetic contributions to IS may clarify the pathophysiology of stroke events without traditional risk factors, and may also lead to the development of novel therapeutic strategies [6]. Most of IS are caused by arterial occlusion secondary to atherosclerosis [7]. It follows that there is emerging interest in the susceptible genes for different pathophysiological pathways of atherosclerosis.

Studies published recently in "Nature" and "Science" journals showed that single nucleotide polymorphisms (SNPs) in Chromosome 9p21.3 region were associated most strongly with coronary artery disease (CAD) [8], which was supported by two meta-analyses [9,10]. Moreover, these SNPs were also associated with peripheral artery disease [11], including intracranial aneurysm [12] and abdominal aortic aneurysm [13]. It has been found that atherosclerotic stroke shared common risk factors and pathophysiological mechanisms with CAD [14], and both diseases appeared to have common susceptibility loci [15]. A few studies based on European ancestry and Chinese Han population also indicated that some sequence variants on chromosome 9p21.3 conferred risk for IS [16–21], especially large-artery stroke (LAA) subtype. The findings were confirmed by meta-analyses with large sample size [22,23]. However, many of the discovered SNP-based susceptibility variants for IS fall in non-coding regions. They are probably only tagging the real functional variants [16]. In addition, traditional association studies based upon SNPs have only accounted for a modest

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proportion of the total genetic variation in most common disease risk. A substantial proportion of the heritability of many diseases remains unexplained [24], which is therefore still a compelling challenge for researchers. Among others, copy number variation (CNV) has been cited as a potential source of this so-called missing heritability [25]. CNV is defined as a segment of DNA that is 1 kb or larger and is present at a variable copy number in comparison with a reference genome [26]. It has been recognized as one of the main genetic factors underlying genomic disorders and other human diseases [27,28]. Compared with SNP, CNV contains more genetic information. It covers up to 12% of the human genome [29], influences gene dosage, and alters gene expression [26,27], and then may result in certain human phenotypic variation and susceptibility to disease [30]. Some specific CNVs have been shown to associate with a wide spectrum of diseases, such as Crohn's disease, systemic lupus erythematosus, amyotrophic lateral sclerosis, autism, schizophrenia, HIV-1 infection, and psoriasis [31]. It is therefore becoming increasingly clear that genetic studies of complex diseases must pay closer attention to the contribution of CNVs.

The majority of the studies on 9p21 to date have focused on either single SNPs or directly genotyped or imputed SNPs in GWAS based on commercial genotyping arrays. Few groups have yet reported on the presence or absence of CNV in the region [8]. Due to a strong ethnic variation in CNVs [32] and possible subtype-specific susceptibility gene for IS [5], as well as a stronger genetic component in IS patients younger than 70 years of age [5]. Therefore, the main focus of the present study was to investigate the possible association between common CNVs exist in the 9p21 region and the risk of atherosclerotic stroke in a population of Han Chinese aged 19–70 years.

2. Materials and methods

2.1. Study population

Study subjects were recruited from consecutive patients with a first-ever IS admitted to the second clinical medical college of the North Sichuan medical college between January 2012 and December 2015. The inclusion and exclusion criteria for cases have been described in detail elsewhere [33]. In brief, this study included only non-relative Han Chinese with atherothrombotic cerebral infarction (ATS, the main IS subtype of the Korean modified TOAST classification) [34], aged 19–70 years within 2 weeks of the event. The patients with IS secondary to other diseases and the ATS cases with liver/kidney disease, systemic infection, thrombolytic therapy, pregnancy or other serious situation were excluded. In addition, unrelated age- and sex-matched healthy controls were randomly selected from volunteers accepting health examination in the same hospital during the same period, who were free of neurological diseases by clinical manifestations and physical examination, following the same exclusion criteria as case subjects. All controls were genetically unrelated Han Chinese.

The study was approved by the Ethics Committee of North Sichuan medical college. All eligible participants provided their signed informed consents.

2.2. Baseline examination

A detailed medical history was obtained from each subject through an interview and physical examination. Demographic features, clinical features, biochemical parameters, and established risk factors for IS were recorded. A blood sample was obtained from all subjects for genetic analysis. A history of coronary heart disease (CHD; i.e., angina pectoris, unstable angina, myocardial infarction, or heart failure), as well as heart valve disease and arrhythmias was assessed by a questionnaire and relevant medical confirmation. Cigarette smoking was defined as having smoked at least one cigarette per day for 1 year or more. Former smokers who had stopped smoking >5 years previously were not included [35]. The presence of alcohol consumption was defined as drinking alcohol at least 12 times during the past year [35]. Hypertension was defined as present if subjects had previously been diagnosed according to the World Health Organization (WHO)/International Society of Hypertension guidelines (systolic or diastolic blood pressure ≥ 140 mmHg or ≥90 mmHg, respectively) [36], and were routinely receiving antihypertensive therapy. Diabetes mellitus (DM) was characterized by recurrent or persistent hyperglycemia, and was diagnosed by the presence of 1) fasting plasma glucose ≥7.0 mM, 2) plasma glucose ≥ 11.1 mM at 2 h after a 75.0 g oral glucose challenge, or 3) random plasma glucose ≥11.1 mM, or if a history of DM was reported (including patients with antidiabetic medication; WHO) [37]. Dyslipidemia was diagnosed using Chinese criteria published in 2007 [38], and the baseline body mass index (BMI) was calculated as the weight divided by the height squared, with a BMI of 24 kg/m^2 taken as the cutoff point for being overweight [39,40].

2.3. DNA isolation and CNVs detection

Venous blood samples were collected from all subjects in EDTA-treated evacuated tubes. Genomic DNA was extracted from blood samples according to standard procedures by using a TIANamp Genomic DNA kit (TIANamp Blood DNA Kit, DP318, TIANGEN Biotech, Beijing, China). All DNA fragments were subjected to electrophoresis on 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide. The copy numbers of the target genes were measured by a custom-by-design Multiplex AccuCopy™ Kit (Genesky Biotechnologies Inc., Shanghai, China) based on a multiplex fluorescence competitive PCR principle as described by Du et al. [41], which can interrogate CNV status at multiple genomic loci in the same assay reaction [41].

The methods below briefly describe the manufacturer's process. A total of 8 target genomic segments within the 4 genes (*ANRIL*, *CDKN2A*, *CDKN2B* and *MTAP*) (two segments for each gene) and 4 reference segments (2p, 10pL, 20q and 16p) were chosen for the AccuCopy assay. The primers for both target and reference segments were synthesized. The forward primers were fluorescent-labeled at Genesky Biotechnologies (Shanghai, China). The competitive DNAs for the eight target and four reference segments were synthesized in double strand and provided in a mixture from Genesky Biotechnologies (Shanghai, China). These competitive DNAs are almost same as their homologies in the human reference genome except 1–2 base pairs deleted. The primers of target segments and reference segments and probes information were provided in Table 1.

The PCR reaction was prepared in 20 μl for each sample, containing a mixture of 2 μl target genomic DNA (5 ng/μl) with 2 μl reference segment DNA, 1 μl Multiplex PCR Fluorescence Primer Mix (AccuCopy™), 10 μl 2× PCR Master Mix (Genesky Biotechnologies), and 5 μl ddH_2O. The program used was an initial denaturation step of 95 °C for 10 min followed by the first 11 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 40 s (the annealing temperature was decreased by 0.5 °C in each consecutive cycle), and elongation at 72 °C for 1.5 min, followed by the second 24 cycles of denaturation at 94 °C for 20 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 1.5 min, and a final extension step at 60 °C for 60 min and then at 4 °C forever. The PCR product was diluted in 1:5 ratio, and 1 μl diluted products was then mixed with 0.5 μl 500 (Liz) size standard and 8.5 μl Hi-Di formamide (both from Applied Biosystems, Foster City, CA). The mixture was subjected to a denaturation step of 95 °C for 5 min, and electrophoresed in 3730XL genetic analyzer (ABI, Carlsbad, CA, USA). Raw data were analyzed by GeneMapper 4.0 (ABI). The height and area data for all specific peaks were exported into a Microsoft Excel file. The sample/competitive (S/C) peak ratio was calculated for all eight target segments and four reference segments. The S/C ratio for each target fragment was first normalized based on four reference segments, respectively. The four normalized S/C ratios were further normalized to the median value in all samples for each reference segment, respectively, and then averaged. If one of the four normalized S/C ratios deviated >25% from the average