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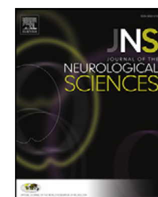

Angiopoietin-like protein 4 serum levels and gene polymorphisms are associated with large artery atherosclerotic stroke

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ABSTRACT

Background: Angiopoietin-like protein 4 (ANGPTL4) is a central player in lipid metabolism and atherosclerosis and may thus be involved in ischaemic stroke. However, no study in humans has investigated the association of ANGPTL4 gene polymorphisms or serum levels with ischaemic stroke.
Methods: We investigated the influence of the tagged single nucleotide polymorphisms (tSNPs) rs4076317 (c.207C>G) and rs1044250 (c.797C>T; T266M) of the ANGPTL4 gene on ischaemic stroke risk in a large group of 712 large artery atherosclerotic (LAA) stroke patients and 828 controls. In addition, we examined the association of the serum ANGPTL4 levels with lipid metabolism, LAA stroke severity and ischaemic volume in a sample of 302 LAA stroke patients and 307 controls.
Results: The findings reveal that rs4076317 exerts a co-dominant effect on lower serum TG levels compared with common homozygotes. Fewer stroke cases were homozygous for variants of rs4076317 compared with the controls (7.0% vs. 10.9%). The serum ANGPTL4 levels in patients were significantly higher than those in the controls in a univariate manner ($P = 0.001$) and after adjustment for other risk factors (1.463 [1.215–1.835]; $P < 0.001$). Consistently, the ANGPTL4 levels were statistically correlated with higher NIHSS scores ($r = 0.172$, $P = 0.003$) and larger lesion volumes ($r = 0.124$, $P = 0.031$).
Conclusion: We concluded that the tagged SNPs and high serum levels of ANGPTL4 are associated with LAA stroke and the lipid characteristics.

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1. Introduction

In recent years, angiopoietin-like 4 (ANGPTL4) is a multifunctional protein that has been implicated in many metabolic and non-metabolic conditions, including energy homoeostasis, angiogenesis, wound repair, redox regulation and tumourigenesis [1].

The human ANGPTL4 gene is located on chromosome 19p 13.3, which has seven exons and encodes a 406-amino-acid glycoprotein with a molecular mass of ~45–65 kDa [1]. Similarly to its family members, ANGPTL4 is cleaved into distinct regions: an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain [2, 3]. Both the N- and C-terminal fragments together with the full-length protein can be detected in the circulation [3]. During evolution, the N- and C-terminal regions were present separately in different organisms, suggesting that the different domains of ANGPTL4 may have distinct physiological functions [2].

Accumulating evidence suggests that ANGPTL4 inhibits lipoprotein lipase (LPL) activity through the N-terminal coiled-coil domain; thus, ANGPTL4 could be associated with lipid metabolism disorders [2–5]. Several studies have shown that the C-terminal fibrinogen-like domain of ANGPTL4 is a master regulator of vascular permeability and angiogenesis [2, 3]. However, the role of ANGPTL4 in vascular angiogenesis remains controversial. Some studies have demonstrated an anti-angiogenic role for this protein [6–8], whereas other studies have shown a pro-angiogenic role for ANGPTL4 [9, 10].

Increasing evidence indicates that ANGPTL4 is involved in the pathophysiology of atherosclerosis [11–13] and may therefore be associated with atherosclerotic diseases, such as coronary heart disease and stroke. Until recently, studies investigating the impact of ANGPTL4 variants or plasma levels on cardiovascular risk have generated conflicting results, showing either an increased or decreased risk of coronary heart disease in patients [11, 14–16]. However, to the best of our knowledge, the association of ANGPTL4 variants or serum levels with ischaemic stroke in humans has not been investigated.

Thus, we explored the relationship between the tagged SNPs and high serum levels of ANGPTL4 and large artery atherosclerotic (LAA) stroke (including the stroke severity and ischaemic volume) and

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determined whether ANGPTL4 plays a role in lipid metabolism in Chinese individuals.

2. Materials and methods

2.1. Study subjects

A total of 712 hospital-based first-time large artery atherosclerotic (LAA) stroke patients were consecutively recruited from the Department of Neurology at Taizhou Hospital of Zhejiang, China, from May 2013 to March 2015. A qualified stroke neurologist examined the LAA stroke patients, and the individuals were differentiated by brain imaging using a computed tomography scan (CT) or magnetic resonance imaging (MRI). As a control group, 828 unrelated age-matched healthy individuals without any neuroimaging modifications were recruited during physical check-ups.

Subjects with any history of cranial trauma, cerebral haemorrhage or aneurysm, serious cardiac disease, recent surgery (3 months), severe liver disease, renal failure, haematologic or autoimmune diseases, chronic inflammatory diseases, history of tumours and inability to undergo CT and MRI imaging were excluded.

The present study was approved by the Medical Ethics Committee of Taizhou Hospital, and all of the subjects provided written informed consent.

2.2. Data collection

All of the participants provided a complete health history and underwent physical examination and laboratory analysis. The patients' age, sex and race were determined from their identity card, and information regarding smoking and drinking status, drug treatment, and history of previous illness was obtained from the participants or their family members. The patients' height and weight were measured to calculate their body mass index (BMI). Their blood pressure was measured three times on the screening day, and the average systolic blood pressure (SBP) and average diastolic blood pressure (DBP) were used in subsequent analyses.

Within 24 h of stroke onset, venous blood samples for all of the individuals were collected after overnight fasting. The fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fibrinogen and homocysteine concentrations were measured at the clinical laboratory in the hospital. Whole blood or serum samples for DNA isolation or the determination of ANGPTL4 levels were stored at −70 °C.

2.3. Assessment of stroke severity and volume

The stroke severity was assessed upon admission using the National Institutes of Health Stroke Scale (NIHSS; scores range from 0 to 42, with higher scores indicating greater deficits) [17].

The infarct volume was evaluated. Among the 302 patients whose ANGPTL4 levels had been determined, 279 and 23 patients were examined by MRI and CT, respectively. MRI (including diffusion weighted imaging [DWI]) was performed within 77 h (median time, 51 h) of disease onset. CT was performed within 90 h (median time, 47 h) of disease onset for patients unable to undergo MRI. A neuroradiologist blinded to the clinical presentations and NIHSS scores of the patients calculated the infarct volume.

2.4. Serum ANGPTL4 levels

The serum ANGPTL4 levels were determined using a commercial ANGPTL4 enzyme-linked immunosorbent assay (ELISA) kit (Raybiotech, Atlanta, GA, USA; Catalogue # ELH-ANGPTL4). According to the manufacturer's information, the minimum detectable overall level of human ANGPTL4 was determined as 20 pg/ml with an intra-assay CV% of <10% and an inter-assay CV% of <12%.

Among the 1540 individuals, the ANGPTL4 levels of 63 samples from patients who received emergency thrombolysis and 37 samples demonstrating gross haemolysis were not determined because these conditions can potentially affect the serum ANGPTL4 concentrations. The serum samples of 609 individuals (307 controls and 302 cases) were randomly selected for determination of the ANGPTL4 levels.

2.5. Selection and genotyping of tagged SNPs

Tagged ANGPTL4 SNPs were selected from the HapMap SNP database [release #27; analysis panel: CHB + JPT (Han Chinese in Beijing, China and Japanese in Tokyo, Japan)] using a minor allele frequency (MAF) of at least 0.01 and a pairwise r^2 of at least 0.8, as determined using the Tagger software [18], implemented in the Haploview programme, as criteria [19]. Thus, each tagged SNP acts as a direct proxy for all other correlated SNPs and, based on the definition, is not highly correlated with other tagged SNPs selected for genotyping. Two tagged SNPs, rs4076317 (c.207C>G) and rs1044250 (c.797C>T; T266M), capturing variation over a region comprising 13 kb of chromosome 19, from position 8,333,011 to 8,345,757 (NCBI build 36), including approximately 2-kb 5′-flanking and 0.5-kb 3′-flanking sequences of the ANGPTL4 gene, were selected for genotyping. Because several effects of rs116843064 (c.118G>A, E40K) have been previously described, we typed rs116843064 in 100 subjects in the present study, but this SNP could not be detected and was not pursued.

The genomic DNA from the whole-blood samples was extracted using the Blood Genomic DNA Isolation Kit (Shanghai Generay Biotech Co., Ltd., Shanghai, China) according to the manufacturer's protocol. The DNA samples obtained from all individuals were available for genotyping. Detailed information on the polymerase chain reaction and DNA sequencing is provided in the appendices.

2.6. Statistical analysis

2.6.1. Demographic and clinical characteristics

The Kolmogorov-Smirnov test was used to test for normality. Continuous variables with a normal distribution are expressed as the means ± SD, whereas those with a skewed distribution are expressed as the medians (25% and 75% quartiles). The variables with a skewed distribution were also normalized through a $\log_{10}$ transformation and are expressed as the geometric means ± approximate SD. All continuous variables were compared using *t*-tests, an analysis of variance (ANOVA) using a post hoc Bonferroni's test among the subgroups, or the rank test, whichever was more appropriate. Categorical variables are expressed as percentages and were compared using Pearson's Chi-squared statistic. Correlation analyses were calculated using either Pearson's correlation coefficients or Spearman's Rank correlation coefficients, whichever was more appropriate. $P < 0.05$ was considered statistically significant.

2.6.2. Genotypic and allelic test of association

The Hardy-Weinberg equilibrium was evaluated among the controls using the Chi-Squared test. The genotype and allelic frequencies between the cases and controls were compared using the Chi-squared test. Assuming a co-dominant inheritance model, indicator variables were generated for each ANGPTL4 genotype frequency using homozygotes for the major allele as the reference category. Multivariable logistic regression models were used to estimate the odds ratios (OR) and 95% confidence interval (CI) for each genotype compared with the wild-type homozygote, with values of 0, 1, and 2 corresponding to the number of minor alleles.

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