Pentanucleotide TTTTA Repeat Polymorphism of Apolipoprotein(a) Gene and Plasma Lipoprotein(a) Are Associated With Ischemic and Hemorrhagic Stroke in Chinese
A Multicenter Case-Control Study in China

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Background and Purpose—It is still inconclusive whether high plasma lipoprotein(a) [Lp(a)] level is a risk factor for stroke. Small sample size and different ethnic groups and methodologies might be contributors to the conflicts in study results. The purpose of the present study was to investigate the association between plasma Lp(a) levels, pentanucleotide TTTTA repeat (PNTR) polymorphism of the apolipoprotein(a) [apo(a)] gene, and Chinese stroke in a case-control study.

Methods—We recruited 1825 cases with stroke (44.3% cerebral atherothrombosis, 28.3% lacunar infarction, and 27.3% intracerebral hemorrhage) and 1817 controls from 7 centers in China. Lp(a) concentrations were quantified by enzyme-linked immunosorbent assay. The PNTR polymorphism of the apo(a) gene was determined by polymerase chain reaction–polyacrylamide gel electrophoresis. Conditional multivariate logistic regression analysis was used to identify independent risk factors for stroke and its subtypes.

Results—Lp(a) levels were significantly higher in cases than in controls (median, 28.5 versus 23.1 mg/dL; \( P < 0.001 \)), leading to a 1.97-fold (95% CI, 1.64 to 2.37) increase in risk for overall stroke, 2.0-fold (95% CI, 1.59 to 2.52) increase for atherothrombotic type, 2.05-fold increase (95% CI, 1.59 to 2.63) for lacunar type, and 1.64-fold increase (95% CI, 1.21 to 2.21) for hemorrhagic type. The number of PNTR negatively correlated with Lp(a) levels. Low-number repeats (sum of both alleles <16) of apo(a) PNTR were associated with both atherothrombotic stroke (odds ratio, 1.41; 95% CI, 1.04 to 1.91) and hemorrhagic stroke (odds ratio, 1.62; 95% CI, 1.09 to 2.37).

Conclusions—Our results indicate for the first time that low numbers of apo(a) PNTR and plasma Lp(a) levels are independently associated with both ischemic and hemorrhagic stroke in Chinese. (Stroke. 2003;34:1617-1622.)

Key Words: apolipoproteins ■ lipoproteins ■ polymorphism ■ stroke
account for 40% to 70% of the interindividual variation in Lp(a) levels. However, recent research argued that such effects might be overestimated. Variations at the apo(a) gene locus beyond the kringle IV-2 might contribute to Lp(a) concentrations. The pentanucleotide TTTTA repeat (PNTR) polymorphism located at the 5’ untranslated region of the apo(a) gene might account for 10% to 14% of the variation in Lp(a) levels and was reported to be associated with IHD independently of apo(a) size polymorphism in whites. However, its association with stroke has not been challenged in any ethnic group thus far. Therefore, in this case-control study, we explored the relationship of Lp(a) levels and the PNTR polymorphism of the apo(a) gene with stroke and its subtypes in Chinese.

Subjects and Methods

Subjects
This is a multicenter study for assessment of risk factors for stroke sponsored by the Ministry of Science and Technology of China (973 Project). The study protocol was reviewed and approved by the review board of Ministry of Public Health, Ministry of Science and Technology of China, and was approved by the ethics committees at all participating hospitals. Informed consent was obtained from all individuals.

Cases and controls were recruited from the same demographic area and at the same time through November 2000 to November 2001 from 7 clinical centers located in Yanzhou, Xi’an, Chongqing, Wuhan, Beijing, and Tianjin. Three subtypes of stroke—cerebral atherosclerosis (atherothrombosis), lacunar infarction (lacunar), and intracerebral hemorrhage (hemorrhage)—were included. Other types of stroke, including transient ischemic attack, subarachnoid hemorrhage, embolic brain infarction, brain tumors, and cerebrovascular malformation, and severe systemic diseases such as collagenosis, endocrine and metabolic disease (except diabetes mellitus [DM]), inflammation, liver, neoplastic, or renal diseases were in the range of exclusion. Diagnosis of stroke was based on the results of strict neurological examination, CT, or MRI according to the International Classification of Diseases, ninth revision. Controls were selected from inpatients (21.5%) with minor illness from the departments of ophthalmology, gastroenterology, otorhinolaryngology, and orthopedics and from community-based inhabitants (78.5%) free of neurological diseases following the same exclusion criteria as cases. At each local community appointed, both men and women 35 to 74 years of age in the range of selection were grouped by age (5-year range for each group), and when 1 case was enrolled, a control was randomly selected from the corresponding group.

Initially, 2000 cases and 2000 controls were enrolled. Before data assessment, we excluded 358 subjects at different experimental stages because of lack of definite diagnosis (24), absence of plasma (76 cases, 93 controls), and failure to detect apo(a) genotypes (75 cases, 90 controls). Complete data were available for analysis for 1825 cases (mean±SD age, 60.3±9.5 years; 63.3% male) and 1817 controls (mean±SD age, 59.6±8.5 years; 59.6% male). Among 1825 cases, 809 (44.3%) had been diagnosed as atherothrombosis, 517 (28.3%) as lacunar, and 499 (27.3%) as hemorrhage. Apart from the neurological history and history of hypertension and DM, the following vascular risk factors for each individual were also recorded: cigarette smoking, body mass index, systolic and diastolic blood pressures, blood glucose, total cholesterol, total triglycerides, high-density lipoprotein cholesterol (HDL-C), and non–HDL-C.

Measurement of Lipids and Lp(a)
Blood samples were drawn into vacuum tubes containing EDTA after a 12-hour overnight fast. In subjects with acute stroke, the drawing of blood was delayed for at least 6 weeks. Plasma biochemical parameters were assayed by an automatic analyzer (7060, Hitachi). Non–HDL-C was calculated by the Friedewald formula. Lp(a) levels were quantified by enzyme-linked immunosorbent assay using coated mouse monoclonal anti-apo(a) antibody and a horseradish peroxidase–labeled rabbit polyclonal anti-apoB antibody for detection. The limit of detection was 0.2 mg/dL, and the intra-assay and interassay coefficients of variation were 5.7% and 7.4%, respectively.

Determination of PNTR of Apo(a) Gene
Genomic DNA was isolated from peripheral blood white cells as described previously. Fragments comprising the pentanucleotide sequence were amplified by polymerase chain reaction (PCR) using the following primers: 5’-ATT TGC GGA AAG ATT GAT ACT ATG-3’ and 5’-GCT ACT AGG GAG GCT GGA GTA TT-3’ on the thermal cycle procedure for 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 51°C, and 40 seconds at 72°C, ending with 7 minutes at 72°C.

The number of TTTTA repeats was determined by size fractionation on nondenaturing 12% polyacrylamide gels. Sizes were estimated by a 5-bp DNA ladder made with a mixture of PCR products of DNA samples containing PNTR genotyped through DNA sequencing (Perkin Elmer, ABI377 DNA Sequencer).

Statistical Analysis
Plasma Lp(a) and triglycerides were highly skewed. The Mann-Whitney method was applied to compare these variables; their logarithmic transformed values were used in multivariate analysis. Departures from Hardy-Weinberg equilibrium and the distribution of PNTR alleles and genotypes of apo(a) gene between groups were tested by χ² test. When only small numbers were available, they were pooled before computing χ². The relation between Lp(a) concentrations and apo(a) genotypes was evaluated by multivariate linear regression. For analysis of association between apo(a) genotype and stroke or subtypes, genotypes were divided into low-repeat-number group (sum of both alleles <16) and high-repeat-number group (sum of both alleles ≥16). Relative risk [represented by the odds ratio (OR) and 95% confidence interval (CI)] analysis was carried out by conditional multivariate logistic regression model controlling for body mass index, systolic and diastolic blood pressures, blood glucose, total cholesterol, total triglycerides, HDL-C, non–HDL-C, and cigarette smoking. All data were analyzed with the SPSS 10.0 package. A value of P<0.05 was considered significant (2 tailed).

Results
Clinical Characteristics
The clinical and demographic features of 3642 subjects are shown in Table 1. As expected, stroke patients had a higher prevalence of conventional vascular risk factors, including history of hypertension and DM, higher levels of plasma triglycerides and glucose, and lower levels of HDL-C, whereas total cholesterol and non–HDL-C levels in patients were surprisingly lower than in controls.

Distribution of PNTR Alleles and Genotypes of Apo(a) Gene
A total of 8 different alleles with the number of TTTTA repeats ranging from 4 to 11 consisting of 30 genotypes were found. Table 2 shows the frequency of PNTR alleles and genotypes. The most common allele had 8 TTTTA repeats, with a frequency of 72.3% in controls and 70.1% in cases. An allele with 9 repeats was also common, with a frequency of...
19.9% in controls and 21.1% in cases. This is the first time that an allele with 4 repeats has been identified, but the frequency is very low (0.74% in controls, 1.02% in cases) (data not shown). For convenience, PNTR alleles were combined into group I with 4 to 7 repeats, group II with 8, group III with 8/8 and 8/9, and those with at least 1 allele 8/8 and 8/9 were the 2 most predominant ones, with a frequency of 53.2% and 27.3% in controls and 50.1% and 28.6% in cases, respectively. When genotypes were combined into 4 groups (group I, those with at least 1 allele <8 repeats; group II, 8/8; group III, 8/9; and group IV, those with both alleles ≥9 repeats, combined with genotypes of 8/10 and 8/11), group I genotypes were more frequent in cases than in controls (12.3% versus 10.2%, P<0.05).

Effect of PNTR Genotype on Lp(a) Levels
A negatively correlative trend of PNTR polymorphism and Lp(a) levels was found in controls although the correlation did not reach statistical significance (r=-0.036, P=0.121), but it was evident in cases (r=-0.076, P=0.001) (data not shown). As shown in Table 3, Lp(a) levels were inversely related to the number of PNTR, until reaching genotype 9/9. Genotype 8/9 had the lowest plasma Lp(a) levels. Considering the influence of both alleles on Lp(a) levels, we took the sum of the PNTR number from both alleles <16 as low-repeat-number group and ≥16 as high-repeat-number group for further analysis.5,20

Lp(a) and PNTR Polymorphism and Stroke
Median Lp(a) levels were significantly higher in cases than in controls (28.5 versus 23.1 mg/dL, P<0.001; Table 4). After controlling for conventional vascular risk factors, Lp(a) was found to cause a 1.97-fold increase in risk of overall stroke (95% CI, 1.64 to 2.37) and was significantly associated with all 3 stroke subgroups (Table 4). When the atherothrombosis and lacunar subtypes were combined as ischemic stroke, the association remained significant (OR, 2.01; 95% CI, 1.71 to 2.52) (data not shown). The low-repeat-number group of apo(a) PNTR was independently associated with overall stroke (OR, 1.32; 95% CI,
In subgroups, the association was found only in hemorrhage (OR, 1.62; 95% CI, 1.09 to 2.37) and atherothrombosis (OR, 1.41; 95% CI, 1.04 to 1.91) (Table 4). As expected, hypertension, triglycerides, and cigarette smoking were risk factors, whereas HDL-C was the strongest protective factor for stroke in this population (data not shown).

**Discussion**

In the present study, we found that plasma Lp(a) levels were independently associated with not only ischemic but also hemorrhagic stroke after adjustment for lipid parameters and other traditional vascular risk factors, leading to a 2-fold increase in risk for Chinese stroke. This was consistent with previous reports among different ethnic groups but contradicted some others. The inconsistency might lie in the distinct populations and small sample size studied. Those studies often incorporated ≤100 cases and a similar number of controls, which might be inadequately powered in association studies, especially in further analyses in subgroups of stroke and multiple testing for disease genetics. A sample involving >1000 individuals in each group might be required to generate robust data. Thus, the large number of patients investigated in this study allowed us to study the role of Lp(a) or other risk factors in different stroke subtypes. In addition to ischemic stroke, we also presented the valuable data that Lp(a) might play an important role in hemorrhagic stroke in Chinese. To the best of our knowledge, this is one of the largest studies on Lp(a) and hemorrhagic stroke thus far.

No association was found between baseline Lp(a) levels and the future risk of stroke during follow-up in whites. However, limitations in these prospective studies cannot be ignored. In the cohort study conducted in Finland, serum samples were stored at −20°C for 14 years before analysis, which might have affected Lp(a) levels, and the number of stroke cases was too small to make further classifications, so these results should be interpreted with caution. Using only men and including cardiac embolic stroke might also be reasons for the negative results obtained by Ridker et al. The follow-up of 2 to 3 years with a limited number of subjects might not be long enough to observe any positive results. Furthermore, older ages at onset might play a role.

Lp(a) values were higher in our present study than reported earlier domestically and in Singapore Chinese, which might be due to different assay methods and subjects. Although it is generally admitted that Lp(a) levels are stable in healthy individuals, controversies exist that pathological state–like inflammation and atherosclerosis may promote an increase in Lp(a) levels. However, very recent studies in human beings and transgenic animals demonstrated vigorously that Lp(a) is a cause of atherosclerosis rather than a consequence. Furthermore, there is evidence that Lp(a) remains stable after inflammation and stress such as exercise and acute ischemic stroke.

It has been suggested that Lp(a) levels in Asians are more susceptible to genetic control. Previously, the PNTR polymorphism of the apo(a) gene was reported to be

### Table 3. Impact of PNTR Genotypes on Lp(a) Levels in Cases and Controls

<table>
<thead>
<tr>
<th>PNTR Genotype</th>
<th>Control</th>
<th>Total</th>
<th>Atherothrombosis</th>
<th>Lacunar</th>
<th>Hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>28.8 (127.6)</td>
<td>34.3 (297.0)†</td>
<td>35.9 (149.0)†</td>
<td>33.1 (112.3)†</td>
<td>32.0 (296.9)†</td>
</tr>
<tr>
<td>II</td>
<td>22.7 (171.2)</td>
<td>28.5 (348.7)†</td>
<td>29.3 (342.7)†</td>
<td>27.9 (176.5)†</td>
<td>27.2 (224.7)†</td>
</tr>
<tr>
<td>III</td>
<td>22.1 (144.0)</td>
<td>26.4 (186.0)‡</td>
<td>27.9 (186.0)‡</td>
<td>25.1 (118.3)‡</td>
<td>25.4 (103.9)‡</td>
</tr>
<tr>
<td>IV</td>
<td>24.6 (113.1)</td>
<td>30.0 (248.6)‡</td>
<td>31.8 (248.6)‡</td>
<td>27.1 (135.8)‡</td>
<td>31.8 (132.5)‡</td>
</tr>
</tbody>
</table>

Values are given as median (range) (mg/dL).

†P<0.01, ‡P<0.001 vs controls.

### Table 4. Impacts of Lp(a) and Low Repeat Number of TTTTA Repeats (Sum of Both Alleles <16) on Risk of Stroke

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median (range)</th>
<th>Adjusted OR (95% CI)</th>
<th>Exposure, %</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1817</td>
<td>23.1 (175.1)</td>
<td>1.00</td>
<td>10.1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Stroke</td>
<td>1825</td>
<td>28.5 (348.7)‡</td>
<td>1.97 (1.64–2.37)‡</td>
<td>12.2</td>
<td>1.23 (1.00–1.51)</td>
<td>1.32 (1.03–1.68)*</td>
</tr>
<tr>
<td>Atherothrombosis</td>
<td>809</td>
<td>29.7 (343.8)‡</td>
<td>2.00 (1.59–2.52)‡</td>
<td>12.6</td>
<td>1.28 (0.99–1.66)</td>
<td>1.41 (1.04–1.91)*</td>
</tr>
<tr>
<td>Lacunar</td>
<td>517</td>
<td>27.2 (176.5)‡</td>
<td>2.05 (1.59–2.63)‡</td>
<td>11.4</td>
<td>1.14 (0.84–1.56)</td>
<td>1.17 (0.82–1.65)</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>499</td>
<td>27.5 (297.1)‡</td>
<td>1.64 (1.21–2.21)‡</td>
<td>12.2</td>
<td>1.24 (0.91–1.68)</td>
<td>1.62 (1.09–2.37)*</td>
</tr>
</tbody>
</table>

LNR indicates low repeat number. Crude OR was calculated through 2×2 cross tabulation, and adjusted OR was obtained on multivariate logistic regression after controlling for age, sex, smoking, body mass index, systolic and diastolic blood pressures, DM, total cholesterol, triglycerides, blood glucose, HDL-C, and non-HDL-C.

*P<0.05; †P<0.01; ‡P<0.001.
inversely correlated with Lp(a) levels and have an independent effect on Lp(a) concentrations. In support of these investigations, we found in Chinese a weakly inverse correlation between the number of TTTTA repeats and Lp(a) levels in both stroke cases and controls, but it was statistically significant only in cases, suggesting that the PNTR polymorphism might play a role in stroke. Further analysis showed that genotypes with a low number of TTTTA repeats had a higher frequency in cases, causing a 1.24-fold increase in risk for overall stroke (data not shown). Our results indicated that individuals carrying alleles of lower repeats were probably more susceptible to stroke.

Genotypes are almost blind to pathological processes such as inflammation and cancer. As pointed out by Amemiya et al, apo(a) isoforms associated with high Lp(a) levels might be predictive of IHD, and we are interested in whether low repeats of apo(a) PNTR are associated with stroke. As expected, a mild yet significant association was found between PNTR genotype and overall stroke when they were divided into low- and high-repeat-number groups according to the sum of both alleles. Similar results were seen in recent studies by Brazier et al and Kalina et al in patients with IHD. The association between low-number repeats of PNTR and stroke was prominent in atherothrombosis and hemorrhage but not lacunar, giving rise to the possibility of PNTR polymorphism as a prognostic factor for some types of stroke in the Chinese subpopulation. It is notable that ORs became statistically significant only after adjustment. Possible reasons might be distributive heterogeneity of other risk factors. However, we cannot exclude the possibility that this association between PNTR polymorphism and stroke might be due to the multivariate modeling process or that it might reflect an allelic association between PNTR and other polymorphisms of the apo(a) gene. Apo(a) size polymorphism has been reported to be associated with ischemic stroke in addition to IHD and other sequence polymorphisms should not be ignored. Because of the limited efficient power of a single polymorphism, establishing haplotypes of the above-mentioned apo(a) polymorphisms is imperative.

Nevertheless, our present study indicated that elevated Lp(a) level is independently associated with both ischemic stroke and hemorrhagic stroke in Chinese. For the first time, an association between stroke and PNTR polymorphism was found in both the atherothrombosis and hemorrhage subtypes. We also found that the apo(a) PNTR polymorphism associated with high levels of Lp(a) might be a predictor of Chinese stroke, especially in identifying hemorrhagic subpopulations at high risk. A prospective study is ongoing to see whether the association between Lp(a) levels, PNTR genotypes, and stroke is a causal one.

Acknowledgments

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References


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