Spon
taneous subarachnoid hemorrhage (SAH) accounts for 1.8%–4% of strokes,10,23 and it has a high morbidity and mortality rate. The incidence rate of spontaneous SAH caused by rupture of intracranial aneurysms (IAs) is 6 in 100,000, although this rate was reported after the 1990s with increasing use of CT scanning.11 Despite advances in diagnosis and treatment, the prognosis for SAH associated with a ruptured IA is poor: half of patients with SAH die, and 20% suffer from permanent disability, with loss of independence.7 The incidence of IA is high; approximately 2% in the general population.8,15 Therefore, the best way to reduce the occurrence of SAH is prevention of the formation, growth, and rupture of IAs.

Although IA has a high prevalence and poor outcome, relatively little is known about the molecular pathogenesis of this disease. It is considered to be a complex disease influenced by the environment and genetic components. Smoking, alcohol abuse, and hypertension are established risk factors for SAH.5,19 However, the contribution of inherited factors cannot be overlooked for SAH.

There is significant evidence to suggest that genetic factors play an important part in the pathogenesis of IAs. Familial IAs have not been found to be rare; they account for 7%–20% of patients with aneurysmal SAH not associated with other heritable connective tissue disorders.18 The risk of having an aneurysm is approximately 4 times higher for a close relative than for someone from the general population.16 Furthermore, genome-wide linkage studies and case-control association studies have identified some genes to be susceptible to IAs, mostly including functional candidate genes coding for structural proteins of the extracellular matrix (ECM).9,17

The association of IA with some heritable disorders of the ECM indicates that disruption of the ECM of the arterial wall is a probable factor in the pathophysiological mechanisms of IAs.17 Histological analysis of ruptured and unruptured IAs suggests that the aneurysmal artery

**Association of Kallikrein gene polymorphisms with sporadic intracranial aneurysms in the Chinese population**

**Laboratory investigation**

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**Object**. Variants of Kallikreins have been shown to be risk factors for intracranial aneurysm (IA) in a Finnish population. In the present study, the authors investigated the correlation between polymorphisms in the Kallikrein gene cluster and IAs in the Chinese population.

**Methods**. The association of Kallikrein variants (rs1722561 and rs1701946) with sporadic IAs was tested in 308 cases and 443 controls. The differences in allelic frequencies between patients and the control group were evaluated with the chi-square test.

**Results**. The C allele of rs1722561 showed a significant reduction in the risk of sporadic IA (OR 0.71, 95% CI 0.53–0.95; p = 0.023). However, no association of the variant rs1701946 with sporadic IA was found (OR 0.78, 95% CI 0.57–1.06; p = 0.115).

**Conclusions**. The variant rs1722561 of Kallikreins might reduce the risk of sporadic IAs among individuals of Chinese Han ethnicity. This study confirms the association between Kallikreins and IAs.

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**KEY WORDS** • intracranial aneurysm • Kallikreins • variant • vascular disorders

Abbreviations used in this paper: DSA = digital subtraction angiography; ECM = extracellular matrix; IA = intracranial aneurysm; LDR = ligase detection reaction; MRA = MR angiography; PCR = polymerase chain reaction; SAH = subarachnoid hemorrhage; SNP = single nucleotide polymorphism.
wall undergoes morphological changes associated with vascular remodeling.\(^{6}\)

The kallikrein (KLK) family, including 15 homologous genes, encodes serine proteases known to be implicated in a wide range of normal physiological processes, from the regulation of cell growth to tissue remodeling. The KLK2 and KLK3 genes can cleave some substrates, such as growth factor binding proteins and components of the basement membrane and/or ECM.\(^{3}\) This characteristic indicates that kallikreins could be associated with vascular remodeling. Furthermore, kallikreins are involved in other processes related to IA rupture, such as regulation of blood pressure, neutrophil chemotaxis, vascular cell growth, and inflammatory cascades.\(^{2}\) Taken together, these previous findings in the KLK genes suggest that they may be biological candidates for susceptibility genes for IAs.

All 15 kallikreins are colocalized at the telomeric end of 19q13. Two genome-wide DNA linkage analyses identified that a susceptibility locus for IAs was 19q13.3 in the Finnish group, which was confirmed in a Japanese sample set.\(^{14,20,22}\) Recently, strong associations between the single nucleotide polymorphisms (SNPs) rs1722561 and rs1701946 in the KLK gene cluster and IA were reported in a Finnish population.\(^{21}\)

Taking into account the genetic heterogeneity between races of people, we genotyped these 2 SNPs (rs1722561 and rs1701946) in Chinese individuals to test whether the associations between these SNPs in the KLK gene cluster and IA in the Chinese population are identical to those in the Finnish population.

### Methods

**Ethics Statement**

The study complied with the Declaration of Helsinki and was approved by the Institutional Review Board and Ethics Committee of Fu Wai Hospital. All participants reported themselves to be of Han nationality, and provided written informed consent.

**Study Participants**

This case-control study comprised 308 patients with sporadic IAs and 443 controls. Patients with IAs were recruited between March 2004 and July 2007 in 3 clinical centers located in Shandong Province (Tai’an, Rizhao, and Jinan). Ruptured IAs were diagnosed based on symptoms suggestive of SAH, subarachnoid blood on CT, and a proven aneurysm at angiography (angiogram, CT, or MR angiography [MRA] or MRI), or at surgery. Unruptured IAs were ascertained by CT, MRA (or MRI), conventional angiography, surgery, or autopsy. All IAs suspected by CT and/or CT angiography were confirmed by digital subtraction angiography (DSA). All cases with confirmed IAs based on DSA study results were retrospectively reviewed by 2 independent observers, a neuroradiologist and a neurosurgeon, who were blinded to the clinical history or the results of any previous imaging studies. All the patients included in this study received either endovascular embolization or a neurosurgical operation after confirmation of IA by DSA.

Controls were ascertained using medical records (no medical history of any type of stroke), clinical interview (no family history of IAs or SAH among first-degree relatives), clinical symptoms, or neuroimaging studies including DSA, 3D CT, MRA, or MRI. A total of 24.8% of controls underwent imaging, and 75.2% of controls were local inhabitants free of any type of stroke or family history of IAs or SAH among first-degree relatives.

Risk factors for IAs and SAH were recorded, including medical and family history, smoking habits, and alcohol consumption. Diagnosis of intracerebral hemorrhage was based on the results of strict neurological examination, CT, MRA, or MRI according to the ICD-9. Diagnosis of hypertension was based on WHO criteria.

**Selection of SNPs**

The variants in Kallikreins were selected based on the variant frequency in the Chinese population, according to the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/SNP/). In the Chinese and Japanese populations, the C allele frequency of rs1722561 was 24.2%, and in Han Chinese from Beijing, China, the C allele frequency of rs1701946 was 38.9%.

**Genotyping of SNPs**

Genomic DNA was extracted from 5 ml of EDTA-anticoagulated peripheral blood as described previously.\(^{12}\)

The SNPs, rs1722561 and rs1701946, were genotyped by a ligase detection reaction (LDR) in Shanghai Biowing Applied Biotechnology Co., Ltd. The primer and probe sequences, and polymerase chain reaction (PCR) and LDR product lengths, of the 2 SNPs are summarized in Table 1. A total of 50 ng of genomic DNA was amplified in 20 µl of Multiplex PCR mixture containing 2 µl 1x buffer, 0.6 µl Mgp++, 2 µl dNTPs, 0.2 µl Taq polymerase, 4 µl 1x Q-solution, 0.4 µl primer mix, and 9.8 µl ddH2O. The PCR conditions were as follows: initial denaturing at 95°C for 15 minutes was followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 65°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The thermal cycler Gene Amp PCR system (model 9600, Perkin Elmer) was used. Further amplification was performed in a 10-µl volume of Multiplex LDR mixture, containing the resultant PCR product of 1 µl (100 ng), 1 µl probe mix, 0.05 µl NEB Taq DNA ligase, and 6.95 µl ddH2O. The LDR conditions included initial denaturing at 95°C for 2 minutes, followed by 35 cycles of denaturing at 94°C for 30 seconds, and annealing at 60°C for 2 minutes. The LDR products (1 µl) were mixed with 1 µl ROX (ABI, Inc.) and 1 µl loading buffer, detected in the ABI PRISM 377 DNA Sequencer, and analyzed with Genemapper (ABI, Inc.). Reproducibility of genotyping was confirmed by sequencing in 100 randomly selected samples, with 99% concordance.

**Statistical Analysis**

The chi-square test was used to test for qualitative variables, genotype/allele frequencies, and Hardy-Weinberg equilibrium test. The effects of the SNPs rs1722561 and rs1701946 of Kallikreins on the risk of IAs were test-
ed with logistic regression analysis. Multivariate logistic regression analyses were adopted to adjust for confounding factors, including age, sex, blood pressure, smoking, and alcohol consumption. The statistical power was calculated with Quanto 1.1. According to a dominant model, the statistical power was higher than 81%, detecting an association at \( p = 0.05 \), with a relative risk of 1.7 for alleles at 30%–50% frequencies, indicating a low risk for false-negative results. All reported \( p \) values were based on 2-sided tests of significance, and \( p \) values < 0.05 were considered as statistically significant. All statistical analyses were completed with Software SPSS 13.0.

**Results**

**Clinical Background of Patients**

A summary of clinical characteristics of the study participants is shown in Table 2. The ratio of women to men was 1.6:1, reflecting the high incidence of aneurysmal SAHs in women. The aneurysm ruptured in 90.9% of patients with IAs, and multiple aneurysms were found in 8.8% of patients. The IAs were located in the posterior communicating artery in 131 patients, in the anterior communicating artery in 93 patients, in the middle cerebral artery in 38 patients, in the anterior cerebral artery in 15 patients, in the posterior cerebral artery in 12 patients, and in other intracranial locations in 19 patients.

**Allelic Association Study With SNPs of Kallikreins**

The distribution of the variants rs1722561 and rs1701946 is shown in Table 3, and it conformed to the Hardy-Weinberg equilibrium in cases and controls. The frequency of the CC+TC genotype of rs1722561 was significantly lower in patients with IAs than in the controls (51.6% vs 60%, \( p = 0.022 \); crude OR 0.71, 95% CI 0.53–0.95). After adjustment for conventional vascular risk factors, including age, sex, blood pressure, smoking, and alcohol consumption, the statistical power was higher than 81%, detecting an association at \( p = 0.05 \), with a relative risk of 1.7 for alleles at 30%–50% frequencies, indicating a low risk for false-negative results. All reported \( p \) values were based on 2-sided tests of significance, and \( p \) values < 0.05 were considered as statistically significant. All statistical analyses were completed with Software SPSS 13.0.

**TABLE 2: Clinical characteristics of patients with IAs and controls**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of Patients w/ IAs (%)</th>
<th>No. of Controls (%)</th>
<th>( p ) Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>total</td>
<td>308</td>
<td>443</td>
<td></td>
</tr>
<tr>
<td>mean age in yrs ± SD</td>
<td>51.4 ± 10.9</td>
<td>51.8 ± 11.1</td>
<td>NS</td>
</tr>
<tr>
<td>sex (M/F)</td>
<td>120:188</td>
<td>175:268</td>
<td></td>
</tr>
<tr>
<td>hypertension</td>
<td>197 of 308 (63.9)</td>
<td>58 of 443 (13.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>smoking</td>
<td>70 of 308 (22.7)</td>
<td>85 of 443 (19.2)</td>
<td>NS</td>
</tr>
<tr>
<td>alcohol consumption</td>
<td>72 of 308 (23.3)</td>
<td>105 of 443 (23.7)</td>
<td>NS</td>
</tr>
<tr>
<td>ruptured IAs</td>
<td>280 of 308 (90.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>multiple IAs</td>
<td>27 of 308 (8.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>site of IAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICA, PCoA</td>
<td>131 of 308 (42.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACA</td>
<td>93 of 308 (30.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA</td>
<td>38 of 308 (12.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACA</td>
<td>15 of 308 (4.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCA</td>
<td>12 of 308 (3.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td>19 of 308 (6.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ACA = anterior cerebral artery; ACA = anterior communicating artery; ICA = internal carotid artery; MCA = middle cerebral artery; NS = not significant; PCA = posterior cerebral artery; PCoA = posterior communicating artery.
† According to chi-square test or Student t-test.
Using unconditional logistic regression analysis, the CC+TC genotype of rs1722561 resulted in a significantly reduced risk of IAs (OR 0.71, 95% CI 0.53–0.95; \( p = 0.023 \)) compared with the TT genotype (Table 3). However, there was no association of the variant rs1701946 with sporadic IAs (OR 0.78, 95% CI 0.57–1.06; \( p = 0.115 \)).

**Discussion**

In this study we found a significant association between IAs and the variant rs1722561 of *Kallikreins*, but with results that were opposite to the previous results from the Finnish and Russian populations. We found that the rs1722561 C allele occurs less frequently in patients with IAs than in controls, and appears to have a protective effect in our populations. Our finding regarding the SNP rs1722561 differs from the deleterious effect of this SNP in Finnish and Russian populations. This discrepancy between studies could be because of ethnic differences in genotype frequencies. The TT genotype had a frequency of 48.4% in the patients with IA in our study population, whereas it was only 26.7% in the case group of combined populations (Finnish and Russian).

We could not replicate the result of the SNP rs1701946 achieved by Weinsheimer et al.;\(^21\) no association of the variant rs1701946 with sporadic IAs was found in our study. However, the negative results for this SNP do not exclude the possibility that *KLK* is a genetic risk factor for sporadic IAs. The conflicting results between these 2 studies are mainly due to the genetic heterogeneity in different ethnic populations. Additionally, the presence of genetic heterogeneity may be relevant in a bias caused by variation in the prevalence of a positive family history between populations, because familial IAs have a higher risk than sporadic IAs.

Besides distinct genotype frequencies, genetic heterogeneity in different ethnic populations causing nonreplication of these 2 variants may also be relevant to the variation in the extent and distribution of linkage disequilibrium.\(^1\)\(^4\) Therefore, we think that these conflicting results may result from the linkage disequilibrium of these 2 variants, with varying functional genes in different populations. The SNP rs1722561 is in linkage disequilibrium with genes having a protective effect on IAs in the Chinese population, whereas it is in linkage disequilibrium with deleterious genes in Finnish and Russian populations. Genes linked with the other SNP, rs1701946, have no effect on IA formation and rupture.

Additionally, our nonreplicated results of these 2 variants (rs1722561 and rs1701946) indicate that the role of KLK appears heterogeneous in populations of different ethnic backgrounds and that its role in aneurysm pathogenesis is therefore probably not a dominant one, which may be accounted for by environment interaction and gene-gene interaction. Besides genetic factors, environmental factors also contribute to the pathological processes of IAs, which can influence the function and expression of genes in an epigenetic manner. In that case the distinct differences in lifestyles and environment between Asians and Westerners could cause the distinctions in function and expression of alcohol consumption, using unconditional logistic regression analysis, the CC+TC genotype of rs1722561 resulted in a significantly reduced risk of IAs (OR 0.71, 95% CI 0.53–0.95; \( p = 0.023 \)) compared with the TT genotype (Table 3). However, there was no association of the variant rs1701946 with sporadic IAs (OR 0.78, 95% CI 0.57–1.06; \( p = 0.115 \)).

**Table 3: Distribution of the kallikrein genotypes in patients with IAs and controls**

<table>
<thead>
<tr>
<th>Variant Group (no.)</th>
<th>No. w/ Genotype (%)</th>
<th>Risk of C Allele</th>
<th>p Value*</th>
<th>ORs (95% CI) for TC+TT</th>
<th>Adjusted p Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC+CC</td>
<td>TC+TT</td>
<td>p Value*</td>
<td>Crude</td>
<td>Adjusted</td>
</tr>
<tr>
<td>rs1722561</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (443)</td>
<td>177 (40.0)</td>
<td>60 (13.5)</td>
<td>0.022</td>
<td>0.71 (0.53–0.95)</td>
<td>0.71 (0.53–0.95)</td>
</tr>
<tr>
<td>IA (308)</td>
<td>149 (48.4)</td>
<td>124 (40.3)</td>
<td>0.71 (0.53–0.95)</td>
<td>0.71 (0.53–0.95)</td>
<td></td>
</tr>
<tr>
<td>control (443)</td>
<td>123 (27.8)</td>
<td>219 (49.4)</td>
<td>0.71 (0.53–0.95)</td>
<td>0.71 (0.53–0.95)</td>
<td></td>
</tr>
<tr>
<td>IA (308)</td>
<td>102 (33.1)</td>
<td>145 (47.1)</td>
<td>0.71 (0.53–0.95)</td>
<td>0.71 (0.53–0.95)</td>
<td></td>
</tr>
</tbody>
</table>

* The p values and crude ORs (95% CIs) were determined using a chi-square test between patients and controls.
† Adjusted ORs (95% CI) and adjusted p values were computed using multivariate logistic regression analyses (adjusted for age, sex, hypertension, cigarette smoking, and alcohol consumption).
Variants of Kallikreins and risk of intracranial aneurysms

KLK8. In addition, the importance of gene–gene interactions for susceptibility to complex disease has been recognized. The implication from our results may be that the role of KLK in aneurysm pathogenesis is probably affected by other genes that are different in populations of different ethnic backgrounds, rather than a dominant model.

Conclusions

Our results indicate that the C allele of the SNP rs1722561 is associated with IAs and decreases the risk of these lesions, but that there is no association between the variant rs1701946 and IAs. These conflicting results can be explained by genetic heterogeneity, environmental interactions, and gene–gene interactions. Although our results differ from those of previous publications, this non-replication does not necessarily imply lack of functional association between Kallikrein and IAs. However, to examine the associations of these 2 variants with IAs and to determine the role of KLK genes in the pathogenesis of IAs, more replication studies across other ethnicities with large sample sizes need to be performed to reduce the effect of ethnic backgrounds.

Acknowledgments

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Disclosure

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Author contributions to the study and manuscript preparation include the following: Conception and design: Chen, Suo. Acquisition of data: Lin, Yu, W Song, Zhu. Drafting the article: Suo. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Chen. Statistical analysis: Chen, Sun. Administrative/technical/material support: W Song, Y Song, Y Zhang, C Zhang. Study supervision: Pang, Hui.

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