Polymorphisms in three genes are associated with hemorrhagic stroke

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Abstract
Background: Multiligand receptor for advanced glycation end products (RAGE), osteoprotegerin, and Golgb1 genes may be implicated in atherosclerosis and vascular diseases. Single nucleotide polymorphisms (SNPs) rs1035798 in RAGE gene, rs2073617 and rs2073618 in TNFRSF11B, and rs3732410 in Golgb1 will be investigated on whether there is an association with hemorrhagic stroke (HS) in Chinese population. Methods: A total of 600 subjects including 199 HS patients and 401 controls were assayed. These samples were divided into two groups: the ≤50 year and >50 year groups. Genotyping of SNPs was determined using the SEQUENOM MassARRAY matrix-assisted laser desorption ionization-time-of-flight–mass spectrometry. The association between genotype and HS risk was evaluated by computing the odds ratio (OR) and 95% confidence interval (CI) with multivariate unconditional logistic regression analyses. Results: Our data showed that in the ≤50 year group, the rs1035798 major allele homozygote C/C in RAGE gene was associated with an increased risk of HS, while Golgb1 rs3732410 minor allele homozygote G/G was associated with a decreased risk of HS. In the >50 year group, the major allele homozygote G/G of rs2073618 was found to be associated with an increased risk of HS. Conclusions: The polymorphisms rs1035798 of RAGE gene, rs2073618 of TNFRSF11B, and rs3732410 of Golgb1 might be involved in the risk of HS at different stage of ages.

Introduction
Stroke is the second leading cause of death in population more than 60 years old and the fifth leading cause of death in people aged 15–59 years old in the world (Johnston et al. 2009). In China, the annual stroke mortality rate has exceeded heart disease to become the leading cause of death and adult disability (Liu et al. 2011). Stroke is divided into two types: ischemic and hemorrhagic stroke, with the latter including intracerebral hemorrhage and subarachnoid. Compared to white populations of European origin, Chinese populations have a higher incidence of stroke overall, a higher proportion of hemorrhagic stroke (HS) (Tsai et al. 2013). The previous studies suggested that at least 30% of strokes in China were HS (Hong et al. 1994). The risk factor profiles and prevention strategies are different for ischemic and hemorrhagic stroke (Leppala et al. 1999).

The multiligand receptor for advanced glycation end products (RAGE, alias AGER) contributes to the pathogenesis of vascular disease (Kaleza et al. 2009; Olsson and Jood 2013). The upregulation of RAGE expression was found in human atherosclerotic plaques and aortic vessels (Rittwar et al. 1995; Cipollone et al. 2003). Variants in the RAGE gene, such as rs1800625, rs1800624, and rs2070600 polymorphisms, had been shown to be associated with diabetic atherosclerosis (Pettersson-Fernholm et al. 2003). In one case–control study, genetic variation rs1035798 SNP in the RAGE gene was observed to be associated with the subtype of small-vessel disease (SVD), but not with overall ischemic stroke (IS) (Olsson and Jood 2013). No study reported the association between rs1035798 SNP and HS.
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Osteoprotegerin (OPG) is a glycoprotein, serving as a soluble decoy receptor for two members of the tumor necrosis factor receptor superfamily: RANKL and TRAIL (receptor activator of nuclear factor-κB ligand and tumor necrosis factor–related apoptosis-inducing ligand) (Bord et al. 2004). OPG inhibits osteoclastogenesis and function of differentiated osteoclasts, thereby preventing bone resorption (Biscetti et al. 2013). Several lines of evidence supported that TNFRSF11B gene product OPG is not only a marker but also a mediator of vascular disease (Jono et al. 2002; Schoppet et al. 2003). Serum OPG concentrations have been found to correlate with progressive atherosclerosis and cardiovascular diseases (Kiechl et al. 2004; Ziegler et al. 2005). Although several studies have investigated OPG gene involved in ischemic stroke (Dichgans 2007; Guldiken et al. 2007; Biscetti et al. 2013), studies on the relation between polymorphisms of OPG and HS are still less.

Golgb1 gene encodes the coat protein 1 (COP1) vesicle tethering factor, Giantin, which is responsible for the phenotypic characteristics including osteochondrodysplasia and plays a pivotal role in multiple aspects of chondrogenesis (Katayama et al. 2011). The Golgb1 rs3732410 mutation is a naturally occurring variant and associated with protection from ischemic stroke (Flanagan et al. 2013). However, no data have been recorded on the relationship between this site and HS.

In the present study, the abovementioned SNPs including rs1035798 in RAGE, rs2073617 and rs2073618 in TNFRSF11B, and rs3732410 in Golgb1 were investigated on the association with HS in Chinese population for the first time.

Materials and Methods

Study subjects

Study subjects were recruited from consecutive hemorrhagic stroke patients and unrelated age-matched healthy controls from the People’s Hospital of Jingjiang City, Jiangsu Province, China, between February 2012 and April 2014. Hemorrhagic stroke included cerebral hemorrhage and subarachnoid hemorrhage (Zhang et al. 2013). Patients with hemorrhage due to trauma, tumor, vascular malformation, and coagulopathy were excluded. Control subjects were recruited from the health examination department of the hospital. These subjects had no clinical or radiological evidence of stroke and other neurological diseases. All subjects were of Han origin and lived roughly within the same geographic region. Sex, age, body mass index (BMI), total cholesterol (TC), triglycerides (TG), and fasting glucose (FG) were collected on entry into the study. Potential vascular risk factors were evaluated, including hypertension, diabetes mellitus, drinking, and smoking. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg according to the World Health Organization criteria. Diabetes mellitus was defined as fasting plasma glucose ≥7.0 mmol/L. Written informed consent was obtained from all subjects before participation. The study protocol was approved by the Research Ethics Committee of the People’s Hospital of Jingjiang City, Jiangsu Province, China.

SNPs selection and genotyping

Single nucleotide polymorphisms rs1035798 in RAGE, rs2073617 and rs2073618 in TNFRSF11B, and rs3732410 in Golgb1 were selected according to the previously published papers (Biscetti et al. 2013; Flanagan et al. 2013; Olsson and Jood 2013). Genomic DNA was extracted from whole blood using the AxyPrep Blood Genomic DNA Miniprep kit (Axygen Biosciences, Union City, CA), and the SNPs were genotyped using the SEQUENOM MassARRAY matrix-assisted laser desorption ionization–time-of-flight–mass spectrometry platform (SEQUENOM, San Diego, CA). Primers for polymerase chain reaction (PCR) were designed using Assay Designer software version 3.0 (SEQUENOM) and synthesized by Sangon Biotech (Shanghai, China), and the primer sequences were showed in Table 1. PCR conditions were as follows: denaturation at 94°C for 15 min, followed by 45 cycles of 20 sec at 94°C, 30 sec at 56°C, 1 min at 72°C, and a final extension of 3 min at 72°C. The final primary PCR reaction mix was treated with shrimp alkaline phosphatase to dephosphorylate unincorporated dNTPs. The iPLEX primer extension reaction was further performed with PCR conditions: denaturation at 94°C for 5 sec, followed by 40 cycles of 94°C for 5 sec, and 52°C for 5 sec and 80°C for 5 sec (5 cycles), 72°C for 3 min, and holding at 4°C. PCR products were purified with resin with procedures followed the iPLEX kit standard protocol (SEQUENOM). The purified extension products were spotted onto a 384-well spectroChip (Affymetrix, USA) by using MassARRAY Nanodispenser and determined with the matrix-assisted laser desorption ionization–time-of-flight–mass spectrometer (MALDI-TOF-MS). Genotype analysis was performed in real time with MassARRAY RT software version 3.1 and analyzed by using the MassARRAY Typer software version 4.0 (SEQUENOM).

Statistical analysis

Statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL). Hardy–Weinberg equilibrium (HWE) was carried out for all SNPs of
samples. The hemorrhagic stroke cases and controls were compared using the $\chi^2$ test, and $P < 0.001$ was considered statistically different. The association between SNPs and hemorrhagic stroke risk was analyzed by computing the odds ratio (OR) and 95% confidence interval (CI) with multivariate unconditional logistic regression analyses, and a two-sided $P < 0.05$ was considered statistically significant.

## Results

### Clinical characteristics of HS patients and controls

A total of 199 HS patients and 401 controls were recruited in the study. These samples were divided into two groups: the $\leq 50$ year and the $> 50$ year groups by age stratification. In the $\leq 50$ year group, in terms of age, gender (men %), body mass index (BMI), triglycerides, and hypertension, there are significant differences between the patients and controls ($P < 0.001$), while no difference between the patients and controls on total cholesterol, fasting glucose, and diabetes was found. In the $> 50$ year group, there are significant differences between the patients and controls in terms of age, gender (men %), body mass index (BMI), fasting glucose, hypertension, and diabetes ($P < 0.001$), while no difference between the HS patients and controls on total cholesterol and triglycerides was found. There are significant differences in certain vascular risk factors between HS patients and control subjects (Obach et al. 2001; Yoshida et al. 2010). Characteristics of the patients and controls are summarized in Table 2.

| Characteristic | $\leq 50$ year group | $> 50$ year group |
|---------------|---------------------|------------------|
| No. of subjects | 33                  | 255              |
| Age (years)   | 46 ± 3.5            | 34 ± 9.3         | 0.001 |
| Gender (% men) | 69.7                | 62.4             | 0.001 |
| BMI (kg/m²)   | 27.1 ± 1.8          | 23.9 ± 1.5       | 0.001 |
| TG (mmol/L)   | 2.1 ± 1.53          | 1.5 ± 0.89       | 0.001 |
| TC (mmol/L)   | 5.0 ± 0.88          | 4.7 ± 0.81       | 0.027 |
| FG (mmol/L)   | 5.6 ± 0.93          | 5.4 ± 1.30       | 0.289 |
| Hypertension (%) | 90.9              | 2.0              | <0.001 |
| Diabetes (%)  | 15.2                | 3.5              | 0.115 |

BMI, body mass index; TG, triglycerides; TC, total cholesterol and FG, fasting glucose. Bold numbers indicate that the differences were statistically significant between the two groups.

### Allele frequencies of SNPs between the HS patients and controls

For four SNPs in the $\leq 50$ year group, allele frequency of $RAGE$ rs1035798 was 4.5% (T) and 95.5% (C) in HS patients and controls, respectively, suggesting that these SNPs were not in linkage disequilibrium.
patients, 13.7% (T) and 86.3% (C) in control; allele frequency of TNFRSF11B rs2073617 was 59.1% (T) and 40.9% (C) in HS patients, 62% (T) and 38% (C) in control; allele frequency of TNFRSF11B rs2073618 was 33.3% (C) and 66.7% (G) in HS patients, 75.5% (G) and 24.5% (C) in controls; allele frequency of Golgb1 rs3732410 was 59.1% (A) and 40.9% (G) in HS patients, 54.1% (A) and 45.9% (G) in controls. Except that there was a significant difference in allele frequencies of RAGE rs1035798 major allele homozygote C/C in Golgb1 gene were associated with more than threefold risk of HS ($\chi^2 = 4.3568, P = 0.0369$; OR = 3.421, 95% CI = 1.010–11.585), and rs2073618 minor allele homozygote G/G of rs2073618 was found to be associated with an increased risk of HS ($\chi^2 = 4.2196, P = 0.04$; OR = 1.607, 95% CI = 1.021–2.528). No association was found for other SNPs between the HS patients and controls ($P > 0.05$). These results indicated that rs1035798 major allele homozygote C/C in RAGE gene were associated with an increased risk of HS, while Golgb1 rs3732410 minor allele homozygote G/G was associated with a decreased risk of HS in younger ages; and TNFRSF11B rs2073618 major allele homozygote G/G was associated with an increased risk of HS in older ages in Chinese population.

### Genotype distribution of SNPs between HS patients and controls

Genotype distributions of SNPs were analyzed between the cases and controls. In the ≤50 year group, the polymorphisms rs1035798 major allele homozygote C/C in RAGE gene were associated with more than threefold risk of HS ($\chi^2 = 4.3568, P = 0.0369$; OR = 3.421, 95% CI = 1.010–11.585), and rs2073618 minor allele homozygote G/G was associated with one-fourth fold risk of HS ($\chi^2 = 4.0998, P = 0.0429$; OR = 0.2459, 95% CI = 0.057–1.06) (Table 4). There was no significant difference in genotype frequencies of TNFRSF11B rs2073617 and rs2073618 between the HS patients and control subjects ($P > 0.05$). In the >50 year group, the genotype frequency of TNFRSF11B rs2073618 was 5.4% (CC), 63.3% (GG), and 31.3% (CG) in HS patients, 6.9% (CC), 51.7% (GG), and 41.4% (CG) in controls. The major allele homozygote G/G of rs2073618 was found to be associated with an increased risk of HS ($\chi^2 = 4.2196, P = 0.04$; OR = 1.607, 95% CI = 1.021–2.528). No association was found for other SNPs between the HS patients and controls ($P > 0.05$). In the current study, we investigated the association between genetic variants in RAGE, TNFRSF11B, and Golgb1 genes and risk of HS. The results showed that rs1035798 C/C in RAGE gene were strongly associated with an increased risk of HS, while the Golgb1 rs3732410 polymorphism was associated with a reduced risk of HS in younger group (≤50 years old), and the TNFRSF11B rs2073618 was associated with an increased the risk of the disease in elder group (>50 years old). This suggests that RAGE rs1035798 and Golgb1 rs3732410 variants play a role at a younger age and TNFRSF11B rs2073618 variant exhibited a role in HS occurrence at an elderly age. To the best of our knowledge, this is the first report to

### Discussion

In the current study, we investigated the association between genetic variants in RAGE, TNFRSF11B, and Golgb1 genes and risk of HS. The results showed that rs1035798 C/C in RAGE gene were strongly associated with an increased risk of HS, while the Golgb1 rs3732410 polymorphism was associated with a reduced risk of HS in younger group (≤50 years old), and the TNFRSF11B rs2073618 was associated with an increased the risk of the disease in elder group (>50 years old). This suggests that RAGE rs1035798 and Golgb1 rs3732410 variants play a role at a younger age and TNFRSF11B rs2073618 variant exhibited a role in HS occurrence at an elderly age. To the best of our knowledge, this is the first report to

### Table 3. Allele frequency of SNPs in HS patients and controls.

| Gene SNPs | ≤50 year group | >50 year group |
|-----------|----------------|---------------|
|           | Cases N (%)    | Control N (%) | $\chi^2$ | $P$ value | OR (95% CI) | Cases N (%) | Control N (%) | $\chi^2$ | $P$ value | OR (95% CI) |
| RAGE      |               |               |         |          |            |               |               |         |          |            |
| rs1035798 | T 3 (0.045)    | 70 (0.137)    | 4.4497  | 0.0349   | 0.299 (0.091–0.979) | 37 (0.111)   | 70 (0.137)    | 0.3349  | 0.5562   | 0.864 (0.532–1.405) |
|           | C 63 (0.955)   | 440 (0.863)   |         |          |            | 295 (0.889)  | 255 (0.873)   | 0.3464  | 0.5562   | 0.864 (0.532–1.405) |
| TNFRSF11B |               |               |         |          |            |               |               |         |          |            |
| rs2073617 | T 39 (0.591)   | 315 (0.620)   | 0.2103  | 0.6466   | 0.885 (0.525–1.492) | 213 (0.645)  | 187 (0.640)   | 0.0172  | 0.8958   | 1.022 (0.736–1.420) |
|           | C 27 (0.409)   | 193 (0.380)   |         |          |            | 117 (0.355)  | 105 (0.360)   | 0.0172  | 0.8958   | 1.022 (0.736–1.420) |
| rs2073618 | C 22 (0.333)   | 125 (0.245)   | 2.3936  | 0.1218   | 1.54 (0.888–2.670)  | 70 (0.211)   | 80 (0.276)    | 3.5758  | 0.0586   | 0.701 (0.485–1.014) |
|           | G 44 (0.667)   | 385 (0.755)   |         |          |            | 262 (0.789)  | 210 (0.724)   | 3.5758  | 0.0586   | 0.701 (0.485–1.014) |
| Golgb1    |               |               |         |          |            |               |               |         |          |            |
| rs3732410 | A 39 (0.591)   | 276 (0.541)   | 0.5833  | 0.445    | 1.225 (0.728–2.061) | 170 (0.512)  | 164 (0.562)   | 1.5362  | 0.2152   | 0.819 (0.597–1.123) |
|           | G 27 (0.409)   | 234 (0.459)   |         |          |            | 162 (0.488)  | 128 (0.438)   | 1.5362  | 0.2152   | 0.819 (0.597–1.123) |

Bold numbers indicate that the differences were statistically significant between the two groups.
Table 4. Genotype distributions of SNPs in HS patients and controls.

| Gene  | Genotype | ≤50 year group | >50 year group | χ² | p value | OR (95% CI) | χ² | p value | OR (95% CI) |
|-------|----------|----------------|---------------|----|---------|-------------|----|---------|-------------|
|       |          | Cases N (%)    | Control N (%) |     |         |             |    |         |             |
| RAGE  | rs1035798| CC 30 (0.909)  | 190 (0.745)   | 131 (0.789) | 114 (0.781) | 3.421       | (1.010–11.585) | 0.457 | (0.612–1.805) |
|       |          | TT 0 (0.000)   | 5 (0.020)     | 2 (0.012)  | 5 (0.034)  |             |    |         |             |
|       |          | CT 3 (0.091)   | 60 (0.235)    | 33 (0.199) | 27 (0.185) | 1.7906      | 0.4085 |
|       |          | TT+CT 3 (0.091)| 65 (0.255)    | 35 (0.211) | 32 (0.219) | 0.032       | 0.858  | 1.051   |
| TNFRSF11B | rs2073617| CC 5 (0.152)   | 31 (0.122)    | 26 (0.158) | 13 (0.089) |             |    |         |             |
|       |          | TT 11 (0.333)  | 92 (0.362)    | 74 (0.448) | 54 (0.370) |             |    |         |             |
|       |          | CT 17 (0.515)  | 131 (0.516)   | 65 (0.394) | 79 (0.541) | 7.6874      | 0.0214 |
|       |          | CC+CT 22 (0.667)| 162 (0.638)   | 91 (0.552) | 92 (0.630) | 1.9771      | 0.1597 | 1.385   | (0.879–2.184) |
| TNFRSF11B | rs2073618| CC 4 (0.121)   | 19 (0.075)    | 9 (0.054)  | 10 (0.069) |             |    |         |             |
|       |          | TT 15 (0.455)  | 149 (0.584)   | 105 (0.633) | 75 (0.517) |             |    |         |             |
|       |          | CG 14 (0.424)  | 87 (0.341)    | 52 (0.313) | 60 (0.414) | 4.2253      | 0.1209 |
|       |          | CC+CG 18 (0.545)| 106 (0.416)   | 61 (0.367) | 70 (0.483) | 4.2196      | 0.04   | 1.607   | (1.021–2.528) |
|        |          | (0.286–1.229) |               |           |           |             |    |         |             |
| Golgb1 | rs3732410| AA 8 (0.242)   | 74 (0.290)    | 45 (0.271) | 43 (0.295) |             |    |         |             |
|       |          | AG 23 (0.697)  | 128 (0.502)   | 80 (0.482) | 78 (0.534) |             |    |         |             |
|       |          | GG 2 (0.061)   | 53 (0.208)    | 41 (0.247) | 25 (0.171) | 2.6785      | 0.262  |
|       |          | AG+AA 31 (0.939)| 202 (0.792)   | 125 (0.753) | 121 (0.829) | 2.6728      | 0.1021 | 1.588   | (0.910–2.770) |
|       |          | (0.057–1.060) |               |           |           |             |    |         |             |

Bold numbers indicate that the differences were statistically significant between the two groups.

demonstrate an association of RAGE rs1035798, Golgb1 rs3732410, and TNFRSF11B rs2073618 SNPs with HS risk. However, TNFRSF11B rs2073617 was not associated with HS, irrespective of age factor.

The human RAGE gene is located in the major histocompatibility complex (MHC) class III region on chromosome 6p21.3, consisting of 11 exons, a 3’ UTR (untranslated region) and a 5’ flanking region which overlaps the 3’ UTR of the PRX2 (Zee et al. 2006; Kalea et al. 2009). Genetic studies have identified that approximately 30 polymorphisms occur in the RAGE gene. The upregulation of RAGE gene expression is a hallmark in vascular disease and therefore genetic variants affecting RAGE mRNA or protein levels may therefore be important disease markers (Kalea et al. 2009). In sex-specific analyses, association to overall IS in women but not in men were observed for rs1035798 (OR = 1.36; 95% CI = 1.05–1.76) and rs1800684 (OR = 0.53, 95% CI = 0.36–0.77) (Olsson and Jood 2013). Further analysis revealed that rs1035798 showed significant association with the subtype of SVD after correction for multiple testing (OR = 1.56; 95% CI = 1.16–2.09), but no association with overall IS. Interestingly, in this study, we found the polymorphism rs1035798 was strongly associated with an increased risk of HS (OR = 3.421, 95% CI = 1.010–11.585) in the ≤50 year group, but not in elder group (>50 years old). Anyway, this is the first time to reveal the correlation between this SNP with HS in Chinese population.

Osteoprotegerin, encoded by TNFRSF11B gene, is a member of the tumor necrosis factor receptor superfamily of cytokine that regulates osteoclastogenesis (Simonet et al. 1997). Cells within the cardiovascular system, such as arterial smooth muscle cells, endothelial cells, and megakaryocytes secreted OPG into the circulation (Malyankar et al. 2000; Bord et al. 2004). OPG-G1181C (rs2073618) is the only polymorphism in a coding region of TNFRSF11B, while OPG-T950C (rs2073617) is located in its promoter region (Tsai et al. 2013). This G/C variation results in lysine/asparagine change in the single peptide of OPG, which may have an effect on intracellular trafficking or export efficiency of the final protein and local concentration of OPG in the vessel wall. Aside from being an important regulating molecule in bone formation and resorption (Ziegler et al. 2005; Strand et al. 2002), OPG also served as a vascular calcification inhibitor (Clancy et al. 2006). Studies showed that OPG was an independent predictor of cardiovascular disease (Nybo and Rasmussen 2008; Omland et al. 2008). The previous study reported that rs2073617 C/C and rs2073618 C/C variant genotypes of the OPG gene were significantly and
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independently associated with the increased risk of ischemic stroke in an Italian population with diabetic patients (Biscetti et al. 2013). Carriers of the OPG-1181 (rs2073618) C/C genotype had a significantly increased risk of intracerebral hemorrhage (ICH), but no associations were found between C/C genotype and ischemic stroke, and nor between the OPG-T950C (rs2073617) and stroke subtypes (Tsai et al. 2013), which is consistent with our observation for this SNP. The current study showed the rs2073618 G/G variant genotype was associated with hemorrhagic stroke in Chinese population after 50 years, but not with the younger population of ≤50 years old.

*Golgb1* gene, located on chromosome 11, encodes Giantin (Katayama et al. 2011). It has been reported that *Golgb1* gene is expressed in cultured chondrocytes (Johansen et al. 2001). Giantin is a Golgi apparatus–associated protein and presents on the Golgi membrane and coat protein 1 (COP1) vesicles. The best characterized function of Giantin is to tether COP1 vesicles to the Golgi apparatus, allowing bidirectional cargo transport through the Golgi stack (Malhotra et al. 1989; Orci et al. 1997). Genome-wide association studies revealed that one mutation in *Golgb1* Y1212C (rs3732410) appeared to be strongly associated with a reduced risk of ischemic stroke in all sickle cell anemia (SCA) patients with stroke against control SCA patients (OR = 0.27; 95% CI = 0.14–0.52) (Katayama et al. 2011). Cerebrovascular disease is perhaps the most devastating complication with SCA for younger patients. This study observed that the *Golgb1* rs3732410 minor allele homozygote G/G was strongly associated with decreased risk of HS (OR = 0.2459, 95% CI = 0.057–1.06; Table 4) in the ≤50 year group. These suggested that *Golgb1* rs3732410 variant had an important role in ischemic or hemorrhagic stroke in younger patients.

**Conclusion**

The current study demonstrated that RAGE rs1035798 and *Golgb1* rs3732410 appeared to be strongly associated with an increased and a decreased risk of HS in the group of ≤50 years old, respectively, and the *TNFRSF11B* rs2073618 variants is a risk factor in the susceptibility of HS in the >50 year group in Chinese population for the first time. The association between *TNFRSF11B* rs2073617 and the risk of HS was not found, irrespective of age stratification. However, further studies in other populations are needed to be warranted.

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**Conflict of Interest**

None declared.

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