Polymorphisms of VEGFA gene and susceptibility to hemorrhage risk of brain arteriovenous malformations in a Chinese population

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Aim: To evaluate the influence of the vascular endothelial growth factor A (VEGFA) polymorphisms on risk of presentation with intracerebral hemorrhage (ICH).

Methods: Nine selected VEGFA single-nucleotide polymorphisms (SNPs) were genotyped in 311 patients with brain arteriovenous malformations (BAVM) in a Chinese population. Associations between individual SNPs/haplotypes and the hemorrhage risk of BAVMs were evaluated using logistic regression analysis.

Results: In the single-locus analysis, rs1547651 was associated with increased risk of ICH (adjusted OR=2.11, 95% CI=1.01–4.42 compared with the AA genotype). In particular, an increased risk for ICH was associated with this variant in female patients (adjusted OR=3.21, and 95% CI=0.99–10.36). Haplotype-based analyses revealed that haplotype ‘GC’ in block 1 and haplotype ‘ACC’ in block 2 were associated with a 30%–38% reduction in the risk of ICH in patients with BAVMs compared to the most common haplotype (Psim=0.033 and Psim=0.005, respectively). The protective effect of haplotype ‘ACC’ in block 2 was more evident in male patients and subjects with BAVMs of a size ≥3 cm (adjusted OR=0.57, 95% CI=0.34–0.97 and adjusted OR=0.57, 95% CI=0.31–0.86, respectively).

Conclusion: The results suggest that VEGFA gene variants may contribute to ICH risk of BAVM.

Keywords: brain arteriovenous malformations; cerebral hemorrhage; vascular endothelial growth factor A (VEGFA); single-nucleotide polymorphism (SNPs)
in the region of the VEGFA. To date, several case-control studies have confirmed the association of VEGFA SNPs with a risk of developing several types of tumors\cite{10-13} and other diseases\cite{14, 15}; however, to our knowledge, there is little data on the role of VEGFA SNPs in relation to ICH risk in patients with BAVMs, in spite of the importance of VEGFA gene in the hemorrhagic tendency of BAVMs. Because of the dearth of knowledge in this area, we evaluated both potential functional SNPs and tag SNPs spanning the VEGFA for effects on the risk of presentation with ICH.

**Materials and methods**

**BAVM sample population**

Using the same recruitment method as described previously\cite{16}, we recruited 311 patients diagnosed with incident BAVM (as demonstrated by pathology or angiography), all of whom were genetically unrelated ethnic Han Chinese. These patients were recruited between January 2004 and December 2007 at Huashan Hospital, Fudan University (Shanghai, China). Patients with a family history or diagnosis of hereditary hemorrhagic telangiectasia (HHT) were excluded. Patients with signs of new intracranial hemorrhage on computed tomography (CT) or magnetic resonance imaging (MRI) were defined as ICH. Patients (symptomatic or not), who had non-hemorrhagic intracranial lesions initially detected by CT scan or MRI and were proven to harbor BAVMs by angiography were coded as unruptured cases. The BAVM size and venous drainage pattern were determined by angiography and were classified using standard guidelines\cite{17}. Each participant provided informed consent, and the studies were approved by the Human Subjects Review Committee of Huashan Hospital, Fudan University.

**Polymorphism selection and genotyping**

We selected tagging SNPs (tSNPs) in the VEGFA gene (6p21.3, NT_007592.14) with genotype data of Han Chinese from the International HapMap Project (HAPMAP), Public Release#20/Phase II on April 7th, 2007 (http://www.hapmap.org). tSNPs were selected to cover the whole VEGFA gene. tSNPs with a minor allele frequency (MAF) greater than 0.05 (based on pairwise LD analysis) were selected to capture unmeasured SNPs with a LD coefficient $D' > 0.8$. In addition, four potentially functional SNPs (rs1547651, rs2010963, rs1413711, and rs3025039) in VEGFA that were identified in previous reports were also included in this study. As a result, nine SNPs of the VEGFA were investigated.

We used white blood cell fractions from whole blood samples for the extraction of genomic DNA using the Qiagen Blood Kit (Qiagen, Chatsworth, CA, USA). Genotyping was performed with the MassARRAY iPLEX platform (Sequenom, San Diego, CA, USA) using an allele-specific MALDI-TOF mass spectrometry assay\cite{18}. Primers for amplification and extension reactions were designed using the MassARRAY Assay Design Version 3.1 software (Sequenom), and SNP genotypes were obtained according to the iPLEX protocol provided by the manufacturer. We examined the quality of the genotyping with a detailed QC procedure that ensured a $>95\%$ successful call rate with duplicate calling of genotypes, internal positive control samples and Hardy-Weinberg Equilibrium (HWE) testing. The consistency rate observed in these duplicated samples was 100%.

**Statistical analyses**

Genotype frequencies in ICH and unruptured cases were compared using a $\chi^2$-test. Estimate odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression with adjustments for age and gender. Akaike’s information criterion (AIC) was employed to determine the best fitting model for each SNP\cite{19}. The issue of multiple tests was controlled with 10000 time permutation tests. Pairwise linkage disequilibrium (LD) among the markers was examined using Lewontin’s standardized coefficient $D'$ and LD coefficient $r^2$\cite{20}. Haplotype blocks were defined by Haploview V4.1, as detailed by Gabriel et al\cite{21}. PHASE 2.0 was used to infer the haplotype frequencies based on the observed genotypes\cite{22}. All the statistical analyses were performed using SPSS17.0 software with two-sided tests and a significance level set at 0.05, unless otherwise indicated.

**Results**

**Characteristics of study participants**

Demographic and clinical characteristics of the patients with BAVMs are shown in Table 1. Of the 311 patients genotyped, 58.2% presented with hemorrhage, and 41.8% presented with unruptured BAVMs. The ages (mean±SD) of the patients were 33±14 years in unruptured cases and 30±15 years in ICH cases, with males accounting for 61.5% of unruptured cases and 55.8% of ICH cases. ICH presentation was not significantly associated with gender ($\chi^2$, $P=0.312$) or age at diagnosis ($t$ test, $P=0.063$), but it was associated with deep venous drainage ($\chi^2$, $P<0.001$) and small BAVM size ($\chi^2$, $P<0.001$).

**Table 1. Demographics and BAVM characteristics.**

|                           | Unruptured | %    | ICH   | %    | Total | %    | $P$   |
|---------------------------|------------|------|-------|------|-------|------|-------|
| Demographics              |            |      |       |      |       |      |       |
| Total BAVM cases          | 130        | 41.8 | 181   | 58.2 | 311   |      |       |
| Age at diagnosis          |            |      |       |      |       |      |       |
| Mean age±SD, year         | 33±14      |      | 30±15 |      | 31±15 | 0.063|       |
| $n$                       | 130        | 181  | 311   |      |       |       |       |
| Gender                    |            |      |       |      |       |      |       |
| Female                    | 50         | 38.5 | 80    | 44.2 | 130   | 0.312|       |
| Male                      | 80         | 61.5 | 101   | 55.8 | 181   |      |       |
| BAVM characteristics      |            |      |       |      |       |      |       |
| BAVM size                 | 19         | 18.6 | 83    | 81.4 | 102   | 7.2×10^{-9} |       |
|                           | 111        | 53.1 | 98    | 46.9 | 209   |      |       |
| Venous drainage           |            |      |       |      |       |      |       |
| Exclusively deep          | 25         | 19.2 | 108   | 59.7 | 133   | 8.78×10^{-24} |       |
| Any superficial           | 105        | 80.8 | 73    | 40.3 | 178   |      |       |
Analysis of single SNPs association with ICH risk in patients with BAVMs

In the single locus analysis, the genotype frequency of the rs1547651 and rs3025030 SNPs was significantly different between unruptured cases and ICH cases ($\chi^2$, uncorrected $P=0.049$ and $P=0.047$, respectively); the association of rs1547651 still remained after the logistic regression analysis was adjusted for age and gender (adjusted OR=2.11; 95% CI=1.01 to 4.42 compared with the AA genotype). The remaining 7 SNPs did not reach the statistically significant level (Table 2).

Table 2. Frequency of the distribution of VEGFA SNP genotypes and their association with risk of hemorrhagic presentation.

| Genetic model | Location in gene region | Genotype   | BAVM presentation | Logistic regression |
|---------------|-------------------------|------------|-------------------|---------------------|
|               |                         |            | Unruptured No (%) | ICH No (%)          | $P$ value | $\chi^2$ test | Adjusted OR (95% CI)$^d$ | $P$ value |
| rs1547651     | Promoter                | A/A        | 115 (91.3%)       | 149 (83.2%)         | 0.049     |            | 1.00 (reference) |          |
|               |                         | A/T        | 11 (8.7%)         | 30 (16.8%)          |           |            | 2.11 (1.01–4.42) | 0.047     |
| rs2010963     | 5' UTR                  | CC         | 40 (32.0%)        | 52 (30.1%)          | 0.260     |            | 1.00 (reference) |          |
| Codominant    |                         | GG         | 66 (52.8%)        | 82 (47.4%)          |           |            | 0.95 (0.56–1.61) | 0.845     |
| Dominant      |                         | GG/CC      | 106 (84.8%)       | 134 (77.5%)         | 0.282     |            | 0.63 (0.34–1.16) | 0.535     |
| rs1413711     | Intron_1                | GG         | 78 (61.4%)        | 98 (54.4%)          | 0.859     |            | 1.00 (reference) |          |
| Codominant    |                         | AG         | 43 (33.9%)        | 73 (40.6%)          |           |            | 1.35 (0.83–2.2)  | 0.221     |
| Dominant      |                         | AA         | 6 (4.7%)          | 9 (5.0%)            |           |            | 1.22 (0.41–3.63) | 0.715     |
| rs833069      | Intron_2                | AA         | 36 (29.8%)        | 44 (25.6%)          | 0.117     |            | 1.00 (reference) |          |
| Codominant    |                         | AG         | 67 (55.4%)        | 88 (51.2%)          |           |            | 1.05 (0.61–1.82) | 0.856     |
| Recessive     |                         | GG         | 18 (14.8%)        | 40 (23.2%)          |           |            | 1.78 (0.87–3.63) | 0.116     |
| rs3024994     | Intron_2                | GG         | 78 (61.4%)        | 98 (54.4%)          | 0.203     |            | 1.00 (reference) |          |
| Codominant    |                         | GA         | 43 (33.9%)        | 73 (40.6%)          |           |            | 1.35 (0.83–2.2)  | 0.221     |
| Recessive     |                         | AA         | 6 (4.7%)          | 9 (5.0%)            |           |            | 1.22 (0.41–3.63) | 0.715     |
| rs3025010     | Intron_5                | TT         | 67 (52.8%)        | 97 (53.9%)          | 0.999     |            | 1.00 (reference) |          |
| Codominant    |                         | GC         | 54 (42.5%)        | 72 (40.02%)         |           |            | 0.92 (0.57–1.47) | 0.715     |
| Recessive     |                         | CC         | 6 (4.7%)          | 11 (6.1%)           |           |            | 1.31 (0.46–3.74) | 0.616     |
| rs3025030     | Intron_7                | GG         | 81 (64.3%)        | 131 (74.0%)         | 0.047     |            | 1.00 (reference) |          |
| Codominant    |                         | GC         | 39 (30.9%)        | 42 (23.7%)          |           |            | 0.70 (0.41–1.18) | 0.181     |
| Log-additive  |                         | CC         | 6 (4.8%)          | 4 (2.3%)            |           |            | 0.42 (0.11–1.55) | 0.194     |
| rs3025035     | Intron_7                | GG         | 95 (75.4%)        | 122 (70.1)          | 0.211     |            | 1.00 (reference) |          |
| Codominant    |                         | GC         | 30 (23.8%)        | 47 (27.0%)          |           |            | 1.19 (0.70–2.03) | 0.523     |
| Recessive     |                         | CC         | 1 (0.8%)          | 5 (2.9%)            |           |            | 3.84 (0.44–33.96) | 0.226     |
| rs3025039     | Exon_8                  | CC         | 83 (67.4%)        | 129 (73.7%)         | 0.188     |            | 1.00 (reference) |          |
| Codominant    |                         | CT         | 35 (28.5%)        | 42 (24.0%)          |           |            | 0.81 (0.48–1.39) | 0.449     |
| Recessive     |                         | TT         | 5 (4.1%)          | 4 (2.3%)            |           |            | 0.53 (0.14–2.06) | 0.36      |
| Log-additive  |                         | –          | –                | –                  |           |            | 1.28 (0.82–1.99) | 0.27      |
Analysis of haplotype association with ICH risk in patients with BAVMs

The reconstructed LD plot of the nine SNPs in the 333 controls was described in our previous report [20]. Three blocks were defined by the nine SNPs in VEGFA. Block 1 covered the region of VEGFA from the 5' UTR to the first intron with a length of 2 kb (SNPs 2–3). Block 2 extended 4 kb (SNPs 4–6) and encompassed the middle part of the gene from intron 2 to intron 5. Block 3 contained the terminal section of the gene, ranging mainly from intron 7 to the 3' UTR (SNPs 7–9, size=1 kb). The overall distribution of haplotypes in block 2 was significantly different between unruptured cases and ICH cases (Table 3). Haplotype-specific analysis revealed the haplotype ‘GC’ in block 1 (Psim=0.033) and the haplotype ‘ACC’ in block 2 (Psim=0.005) correlated with a significant protective effect against ICH risk in patients with BAVMs (adjusted OR=0.70; 95% CI=0.49–1.02 and adjusted OR=0.62, 95% CI=0.40–0.93, respectively) compared to the most common haplotype.

Association analysis with stratification

We further evaluated whether the rs1547651 variant and haplotype ‘ACC’ in block 2 were associated with ICH risk in patients with BAVMs, as stratified by age, sex, BAVM size and venous drainage status. As shown in Table 4, compared with the common wild-type homozygous genotype, the increased risk associated with genotype AT of rs1547651 was more pronounced in female subjects (adjusted OR=3.21, 95% CI=0.99–10.36). Moreover, the protective effect of haplotype ‘ACC’ in block 2 was more evident in males and subjects with BAVMs of a size ≥3 cm (adjusted OR=0.57, 95% CI=0.34–0.97 and adjusted OR=0.57, 95% CI=0.31–0.86, respectively).

Table 3. Frequency of the distribution of haplotypes in the VEGFA gene and their association with risk of hemorrhagic presentation.

| Block | Haplotype | Total | ICH (%) | Unruptured (%) | P value | Psim valueb | Adjusted OR (95% CI)c | P valued |
|-------|-----------|-------|---------|---------------|---------|-------------|----------------------|---------|
| Block 1 | CC        | 277   | 168     | 46.4          | 41.9    | 0.277       | 0.283                | 1.00 (reference) |
|        | GC        | 199   | 103     | 28.5          | 36.9    | 0.030       | 0.033                | 0.70 (0.49–1.02) |
|        | GA        | 146   | 91      | 25.1          | 21.2    | 0.288       | 0.324                | 1.09 (0.72–1.65) |
|        |           |       |         |               |         | 0.662       | 0.665                | 1.00 (reference) |
|        | Global-stat=4.79 | df=2 | P=0.091 |              |         |             |                      |         |
| Block 2 | GCT       | 285   | 174     | 48.1          | 111     | 0.115       | 0.114                | 1.00 (reference) |
|        | ACT       | 160   | 94      | 26            | 26.4    | 0.993       | 0.985                | 0.92 (0.62–1.37) |
|        | ACC       | 141   | 69      | 19.1          | 19.1    | 0.007       | 0.005                | 0.62 (0.41–0.93) |
|        | Othersd   | 36    | 25      | 6.9           | 11      | 0.424       | 0.231                | 1.43 (0.68–3.07) |
|        |           |       |         |               |         |             |                      |         |
|        | Global-stat=9.32 | df=4 | P=0.054 |              |         |             |                      |         |
| Block 3 | GCC       | 428   | 252     | 69.8          | 67.7    | 0.662       | 0.665                | 1.00 (reference) |
|        | CCT       | 100   | 51      | 14.1          | 18.8    | 0.105       | 0.117                | 0.75 (0.48–1.17) |
|        | GTC       | 89    | 57      | 15.7          | 12.3    | 0.165       | 0.169                | 1.23 (0.76–1.99) |
|        | Othersd   | 5     | 2       | 0.6           | 1.2     | 0.328       | 0.288                | 0.48 (0.08–2.95) |
|        |           |       |         |               |         |             |                      |         |
|        | Global-stat=5.01 | df=4 | P=0.286 |              |         |             |                      |         |

a Polymorphic bases were in 5'–3' order, as listed in Table 2. Loci chosen for block 1: SNPs 2–3, Loci chosen for block 2: SNPs 4–6, Loci chosen for block 3: SNPs 7–9.
b Generated by permutation test with 10 000 times simulation.
c Adjusted for age and gender.
d Haplotypes with a frequency of less than 0.1 were pooled into one mixed group.

Discussion

VEGFA has the ability to increase vascular permeability and cause vasodilatation, and it has been shown to be associated with various hemorrhagic disorders. Overexpression of VEGFA was reported recently in patients with brain tumor-associated ICH [23]. In this study, we reported the association between multiple common VEGFA polymorphisms and the hemorrhagic risk of BAVMs in a Han Chinese population. We found that one SNP out of nine selected SNPs showed a significant association with ICH risk. Moreover, haplotype analyses revealed that the haplotype ‘GC’ in block 1 (Psim=0.033), and the haplotype ‘ACC’ in block 2 (Psim=0.005) showed a decreased ICH risk in patients with BAVMs compared with those of the common haplotype. Our findings suggested that VEGFA gene variants might contribute to an increased ICH risk in patients with BAVMs.

Genetic variants within the conventional regulatory region, such as the 5' UTR and the 3' UTR, were analyzed as a priority in several previous studies. rs1547651, which is located in the promoter region, was reported to be significantly associated with bladder cancer; moreover, the TT genotype was linked to a three-fold increased risk for bladder cancer [24]. In our study, the heterozygous genotype was significantly associated with increased ICH risk (adjusted OR=2.11, 95% CI =1.01–4.42) compared with the AA genotype. Moreover, our stratified analyses revealed that the AT genotype had a three-fold increased risk of ICH compared with the AA genotype in females (adjusted OR=3.21, 95% CI=0.99–10.36). Despite the fact that the single SNP association of rs1547651 was not significant after using the stringent Bonferroni correction, the association might still be noteworthy. Using the TFSEARCH
program (http://mbs.cbrc.jp/research/db/TFSEARCH.html), we found that two possible transcriptional factors, GATA-1 and GATA-3, could bind to the A allele of rs1547651. We hypothesized that this polymorphism variation may affect the expression of \textit{VEGFA} by changing the binding affinity of factors to the mRNA.

Increasing evidence indicates the importance of intronic \textit{VEGFA} polymorphisms as markers of disease susceptibility\cite{24–26}. One Canadian study suggested that rs3025030 was associated with a higher risk of retinopathy. In our study, the genotype distribution of rs3025030 was significantly different between cases and controls ($P$=0.047). Furthermore, no association was found via logistic regression analyses after adjustment for age and sex. Although the variants of rs3025030 were predicted to cause changes in the binding sites of transcription factors, which may result in the dysfunction of \textit{VEGFA} expression using FASTSNP\cite{27}, the effects of this variant needed to be validated in further studies.

Because we were confident that haplotype-based analysis is more powerful than single-marker analysis\cite{28, 29}, we performed this type of analysis to elucidate which haplotype was associated with an increased or a diminished risk of ICH. Although a few studies have been conducted regarding the involvement of \textit{VEGFA} haplotypes in certain diseases\cite{30-32}, the haplotypes analyzed in those studies are not comparable with ours because they genotyped different SNPs. In our study, we found that the ‘GC’ haplotype in block 1 and the ‘ACC’ haplotype in block 2 were significantly associated with ICH risk (adjusted OR=0.70, 95% CI=0.49–1.02, $P_{\text{sim}}=0.033$ and adjusted OR=0.62, 95% CI=0.41–0.93, $P_{\text{sim}}=0.005$, respectively); moreover, we observed a protective effect of the haplotype ‘ACC’ derived from rs833069, rs3024994, and rs3025010 in females and those with BAVMs of a size $\geq$3 cm. We did not, however, see an independent association with the individual SNP present in the haplotype associated with ICH risk. Our hypothesis is that a combined haplotype, rather than a single SNP, is important for ICH.

Despite the fact that our study has several strengths, including a haplotype-based design and a homogeneous population of the same ethnicity, some inherent limitations must be noted. Some selection bias due to hospital-based controls cannot be ruled out. To limit the potential selection bias, we recruited patients by matching the controls to the individuals with BAVMs on the categories of age, sex, and residential area. Only nine out of the exhaustive list of SNPs in \textit{VEGFA} were genotyped in this study, and therefore, it is possible that we did not fully capture or represent the genetic variability of the gene; however, SNPs with high priority were selected for this

\begin{table}[h]
\centering
\caption{Stratified analyses of the associations between the \textit{VEGFA} rs1547651 genotypes and haplotypes in block 2 with risk of hemorrhagic presentation by selected variables.}
\begin{tabular}{llllll}
\hline
Variables & rs1547651 & & & Block2 & \\
 & Unruptured/ICH & OR (95% CI)$^a$ & & Unruptured/ICH & OR (95% CI)$^a$ \\
 & & & & & \\
\hline
Age at diagnosis & & & & & \\
>30 & 63 (87.5)/7 (9.7)/ & 1.00 (reference) & 1.96 (0.74–5.15) & 61 (42.4)/ & 1.00 (reference) \\
& 68 (81.9) & & & 42 (29.2)/ & 0.59 (0.33–1.05) \\
\leq 30 & 52 (89.7)/4 (6.9)/ & 1.00 (reference) & 2.42 (0.76–7.73) & 50 (43.1)/ & 1.00 (reference) \\
& 81 (82.7) & & & 30 (25.9)/ & 0.66 (0.37–1.20) \\
Gender & & & & & \\
Female & 44 (88.0)/ & 1.00 (reference) & 3.21 (0.99–10.36) & 43 (43.0)/ & 1.00 (reference) \\
& 63 (78.8) & & & 27 (27.0)/ & 0.68 (0.35–1.29) \\
Male & 71 (88.8)/ & 1.00 (reference) & 1.53 (0.58–4.04) & 68 (42.5)/ & 1.00 (reference) \\
& 86 (85.1) & & & 45 (28.1)/ & 0.57 (0.34–0.97) \\
BAVM size & & & & & \\
<3 cm & 17 (89.5)/ & 1.00 (reference) & 2.11 (0.43–10.35) & 16 (42.1)/ & 1.00 (reference) \\
& 66 (79.5) & & & 23 (23.7)/ & 0.90 (0.36–2.28) \\
\geq 3 cm & 98 (88.3)/ & 1.00 (reference) & 1.95 (0.80–4.72) & 95 (42.8)/ & 1.00 (reference) \\
& 83 (84.7) & & & 63 (28.4)/ & 0.52 (0.31–0.86) \\
Venous drainage & & & & & \\
Exclusively deep & 23 (92.0)/ & 1.00 (reference) & 2.88 (0.62–13.4.2) & 22 (44.0)/ & 1.00 (reference) \\
& 86 (79.6) & & & 13 (26.0)/ & 0.63 (0.29–1.40) \\
Any superficial & 92 (87.6)/ & 1.00 (reference) & 1.52 (0.58–4.01) & 89 (42.4)/ & 1.00 (reference) \\
& 63 (86.3) & & & 59 (28.1)/ & 0.62 (0.36–1.06) \\
\hline
\end{tabular}
\footnotesize{$^a$Adjusted for age and gender.}
\end{table}
study based on a careful review of previous functional analyses and association studies of VEGFA variation.

In conclusion, the results from our case-control study in a Chinese population suggest that the genetic variants of the VEGFA gene may modulate ICH risk in patients with BAVMs. In particular, we found two haplotypes with a significantly protective effect with respect to ICH risk in patients with BAVMs. Large-scale studies with ethnically diverse populations and functional evaluation of these studies are warranted to confirm our findings.

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Author contribution
Dr Hong-yan CHEN and Dr Yao ZHAO designed the research and revised the paper; Zhi-ping GONG conducted the experiments and wrote the paper; Ni-dan QIAO, Yu-xiang GU, Jian-ping SONG, Pei-liang LI, and Hui-jia QIU performed the experiments; Wei-wei FAN analyzed the data; and Ying MAO designed the research.

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