Original Article

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 ($P_{\text{sim}}=0.033$ and $P_{\text{sim}}=0.005$, 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)

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Introduction

Brain arteriovenous malformations (BAVMs) are a common cause of intracerebral hemorrhage (ICH), especially in young adults^[1]. An accurate estimate of ICH risk in patients harboring BAVMs is needed to help guide clinical management. Although features of BAVM associated with ICH have been identified, additional robust and reproducible indices are still needed. Genetic variation may influence the pathogenesis and the clinical course of BAVMs. The identification of genetic polymorphisms associated with clinical manifestations would facilitate risk stratification as well as illuminate the underlying

biology^[2].

Vascular endothelial growth factor A (VEGFA) plays an important role in the migration, proliferation and survival of endothelial cells^[3, 4]. It has been hypothesized to be involved in the pathogenesis of cancer metastasis, retinopathy, age-related macular degeneration, rheumatoid arthritis and psoriasis^[5–7]. Molecular characterization of BAVM tissue has provided evidence for the involvement of angiogenesis, and Hashimoto *et al*^[8] have shown that a prominent feature of the BAVM tissue is relative overexpression of VEGFA at both the mRNA and protein levels. Furthermore, VEGFA may contribute to the hemorrhagic tendency of BAVMs, as extrapolated from animal models^[9].

The VEGFA gene is located on chromosome 6p21.3 and comprises a 14 kb coding region with eight exons. Over thirty single nucleotide polymorphisms (SNPs) have been described

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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^[10–13] and other diseases^[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^[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^[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 minimum $r^2 > 0.8$. 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^[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 χ^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^[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 ^[20]. Haplotype blocks were defined by Haploview V4.1, as detailed by Gabriel *et al*^[21]. PHASE 2.0 was used to infer the haplotype frequencies based on the observed genotypes^[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 $\pm$ SD) of the patients were 33 $\pm$ 14 years in unruptured cases and 30 $\pm$ 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 (χ^2 , $P=0.312$) or age at diagnosis (t test, $P=0.063$), but it was associated with deep venous drainage (χ^2 , $P<0.001$) and small BAVM size (χ^2 , $P<0.001$).

Table 1. Demographics and BAVM characteristics.

	Unruptured	%	ICH	%	Total	<i>P</i>
Demographics						
Total BAVM cases	130	41.8%	181	58.2%	311	
Age at diagnosis						
Mean age $\pm$ SD, year	33 $\pm$ 14		30 $\pm$ 15		31 $\pm$ 15	0.063
<i>n</i>	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 $\times$ 10 ⁻⁹
	111	53.1%	98	46.9%	209	
Venous drainage						
Exclusively deep	25	19.2%	108	59.7%	133	8.78 $\times$ 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 (χ^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		<i>P</i> value χ^2 test	Logistic regression	
			Unruptured No (%)	ICH No (%)		Adjusted OR (95% CI) ^a	<i>P</i> value
Genotype: 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		CC	40 (32.0%)	52 (30.1%)		1.00 (reference)	
Codominant	5' UTR	CG	66 (52.8%)	82 (47.4%)	0.260	0.95 (0.56–1.61)	0.845
		GG	19 (15.2%)	39 (22.5%)		1.54 (0.77–3.07)	0.221
Dominant		CG/CC	106 (84.8%)	134 (77.5%)		0.63 (0.34–1.16)	0.535
rs1413711	Intron_1	GG	78 (61.4%)	98 (54.4%)	0.282	1.00 (reference)	
Codominant		GA	43 (33.9%)	73 (40.6%)		1.35 (0.83–2.2)	0.221
		AA	6 (4.7%)	9 (5.0%)		1.22 (0.41–3.63)	0.715
Dominant	Intron_2	GA/AA	49 (38.6%)	82 (45.6%)	0.117	0.75 (0.47–1.19)	0.22
rs833069		AA	36 (29.8%)	44 (25.6%)		1.00 (reference)	
Codominant		AG	67 (55.4%)	88 (51.2%)		1.05 (0.61–1.82)	0.856
	Intron_2	GG	18 (14.8%)	40 (23.2%)	0.203	1.78 (0.87–3.63)	0.116
Recessive		AA/AG	103 (85.1%)	132 (76.7%)		1.00 (reference)	
		G/G	18 (14.9%)	40 (23.3%)		0.58 (0.31–1.08)	0.081
rs3024994	Intron_2	CC	116 (91.3%)	153 (86.9%)	0.203	1.00 (reference)	
Codominant		CT	11 (8.7%)	22 (12.5%)		1.51 (0.70–3.26)	0.298
		TT	0 (0%)	1 (0.6%)		1.25E9 (0.0–)	1
Log-additive	Intron_5	–	–	–	0.999	0.62 (0.30–1.30)	0.2
rs3025010		TT	67 (52.8%)	97 (53.9%)		1.00 (reference)	
Codominant		TC	54 (42.5%)	72 (40.02%)		0.92 (0.57–1.47)	0.715
	Intron_7	CC	6 (4.7%)	11 (6.1%)	0.047	1.31 (0.46–3.74)	0.616
Recessive		TT/TC	121 (95.3%)	169 (93.9%)		1.00 (reference)	
		CC	6 (4.7%)	11 (6.1%)		0.74 (0.26–2.06)	0.55
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
		CC	6 (4.8%)	4 (2.3%)		0.42 (0.11–1.55)	0.194
Log-additive	Intron_7	–	–	–	0.211	1.47 (0.96–2.26)	0.077
rs3025035		CC	95 (75.4%)	122 (70.1)		1.00 (reference)	
Codominant		TC	30 (23.8)	47 (27.0%)		1.19 (0.70–2.03)	0.523
	Exon_8	TT	1 (0.8%)	5 (2.9%)	0.188	3.84 (0.44–33.96)	0.226
Recessive		CC/CT	125 (99.2%)	169 (97.1%)		1 (reference)	
		TT	1 (0.8%)	5 (2.9%)		0.27 (0.03–2.40)	0.19
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
		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^[16]. 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 ($P_{\text{sim}}=0.033$) and the haplotype 'ACC' in block 2 ($P_{\text{sim}}=0.005$) 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=0.4–0.93, 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).

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 ($P_{\text{sim}}=0.033$), and the haplotype 'ACC' in block 2 ($P_{\text{sim}}=0.005$) 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

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

Block	Haplotype ^a	Total	ICH	(%)	Unruptured	(%)	<i>P</i> value	<i>P</i> _{sim} value ^b	Adjusted OR (95% CI) ^c	<i>P</i> value ^c
Block 1	CC	277	168	46.4	109	41.9	0.277	0.283	1.00 (reference)	
	GC	199	103	28.5	96	36.9	0.030	0.033	0.70 (0.49–1.02)	0.063
	GA	146	91	25.1	55	21.2	0.288	0.324	1.09 (0.72–1.65)	0.692
	Global-stat=4.79			<i>df</i> =2		<i>P</i> =0.091		<i>P</i> _{sim} ^b =0.089		
Block 2	GCT	285	174	48.1	111	42.7	0.115	0.114	1.00 (reference)	
	ACT	160	94	26	66	25.4	0.993	0.985	0.92 (0.62–1.37)	0.672
	ACC	141	69	19.1	72	27.7	0.007	0.005	0.62 (0.41–0.93)	0.020
	Others ^d	36	25	6.9	11	4.2	0.243	0.231	1.43 (0.68–3.07)	0.341
Block 3	Global-stat=9.32			<i>df</i> =4		<i>P</i> =0.054		<i>P</i> _{sim} ^b =0.043		
	GCC	428	252	69.8	176	67.7	0.662	0.665	1.00 (reference)	
	CCT	100	51	14.1	49	18.8	0.105	0.117	0.75 (0.48–1.17)	0.202
	GTC	89	57	15.7	32	12.3	0.165	0.169	1.23 (0.76–1.99)	0.393
Block 3	Others ^d	5	2	0.6	3	1.2	0.328	0.288	0.48 (0.08–2.95)	0.428
	Global-stat=5.01			<i>df</i> =4		<i>P</i> =0.286		<i>P</i> _{sim} ^b =0.296		

^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.

Table 4. Stratified analyses of the associations between the *VEGFA* rs1547651 genotypes and haplotypes in block 2 with risk of hemorrhagic presentation by selected variables.

Variables	rs1547651				Block2			
	Unruptured/ICH		OR (95% CI) ^a		Unruptured/ICH		OR (95% CI) ^a	
	AA N _Q (%)	AT N _Q (%)	AA	AT	GCT N _Q (%)	ACC N _Q (%)	GCT	ACC
Age at diagnosis								
>30	63 (87.5)/ 68 (81.9)	7 (9.7)/ 15 (18.1)	1.00 (reference)	1.96 (0.74–5.15)	61 (42.4)/ 77 (46.4)	42 (29.2)/ 32 (19.3)	1.00 (reference)	0.59 (0.33–1.05)
≤30	52 (89.7)/ 81 (82.7)	4 (6.9)/ 15 (15.3)	1.00 (reference)	2.42 (0.76–7.73)	50 (43.1)/ 97 (49.5)	30 (25.9)/ 37 (18.7)	1.00 (reference)	0.66 (0.37–1.20)
Gender								
Female	44 (88.0)/ 63 (78.8)	4 (8.0)/ 17 (21.3)	1.00 (reference)	3.21 (0.99–10.36)	43 (43.0)/ 75 (46.0)	27 (27.0)/ 30 (18.8)	1.00 (reference)	0.68 (0.35–1.29)
Male	71 (88.8)/ 86 (85.1)	7 (8.8)/ 13 (12.9)	1.00 (reference)	1.53 (0.58–4.04)	68 (42.5)/ 99 (49.0)	45 (28.1)/ 39 (19.3)	1.00 (reference)	0.57 (0.34–0.97)
BAVM size								
<3 cm	17 (89.5)/ 66 (79.5)	2 (10.5)/ 15 (18.1)	1.00 (reference)	2.11 (0.43–10.35)	16 (42.1)/ 79 (47.6)	9 (23.7)/ 36 (21.7)	1.00 (reference)	0.90 (0.36–2.28)
≥3 cm	98 (88.3)/ 83 (84.7)	9 (8.1)/ 15 (15.3)	1.00 (reference)	1.95 (0.80–4.72)	95 (42.8)/ 95 (48.5)	63 (28.4)/ 33 (16.8)	1.00 (reference)	0.52 (0.31–0.86)
Venous drainage								
Exclusively deep	23 (92.0)/ 86 (79.6)	2 (8.0)/ 20 (18.5)	1.00 (reference)	2.88 (0.62–13.4.2)	22 (44.0)/ 104 (48.1)	13 (26.0)/ 40 (18.5)	1.00 (reference)	0.63 (0.29–1.40)
Any superficial	92 (87.6)/ 63 (86.3)	9 (8.6)/ 10 (13.7)	1.00 (reference)	1.52 (0.58–4.01)	89 (42.4)/ 70 (47.9)	59 (28.1)/ 29 (19.9)	1.00 (reference)	0.62 (0.36–1.06)

^a Adjusted for age and gender.

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 *VEGFA* by changing the binding affinity of factors to the mRNA.

Increasing evidence indicates the importance of intronic *VEGFA* polymorphisms as markers of disease susceptibility^[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 *VEGFA* expression using FASTSNP^[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^[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 *VEGFA* haplotypes in certain diseases^[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 ≥ 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 *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

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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