Gene 523 (2013) 195–198

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Short communication

Association study between of Tie2/Angiopoietin-2 and VEGF/KDR pathway gene polymorphisms and vascular malformations

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ARTICLE INFO

Article history:
Accepted 19 February 2013
Available online 6 April 2013

Keywords:
Vascular malformations
Tie2
KDR
ANTXR1
Silent SNPs

ABSTRACT

Vascular malformations (VMs) are common congenital and neonatal dysmorphogenesis. VMs mostly occur sporadically with a few exceptions of inheritability. Tie2/angiopoietins-2 (Ang-2) and VEGF/KDR pathways are known to be involved in normal and pathogenic angiogenesis. Our study was aimed to test the contribution of these pathway gene variants to VMs. A total of 8 variants were found among 103 VM patients and 142 healthy controls. These variants comprised rs638203, rs639225, rs80338908 and rs80338909 in Tie2 gene, rs1870377 and rs2305949 in KDR gene, rs79337921 and rs34590960 in ANTXR1 gene. Our results indicated that rs638203 ($p = 0.029$) and rs639225 ($p = 0.018$) in Tie2 gene were associated with VM. A further bioinformatics analysis suggested the rs638203-G and rs639225-G might cause an abnormal splicing of Tie2 gene into to a defective protein. Our results identified two novel Tie2 gene polymorphisms with genetic susceptibility to VMs, although future functional validation of the two polymorphisms is warranted in the future.

1. Introduction

Vascular malformations (VMs), common congenital and neonatal dysmorphogenesis, can be subdivided into low-flow lesions (capillary, lymphatic, and venous malformations) and high-flow lesions (arteriovenous malformations and arteriovenous fistulae) according to the biological classification developed by Mulliken and Glowacki in 1982. VMs usually occur sporadically; however there are rare familial cases such as cutaneomucosal venous malformations (Gallione et al., 1995; Pasyk et al., 1984). VMs can occur in any organ, the head and neck are the most common location. Some individuals had VMs located in internal organs, including pulmonary, gastrointestinal, renal, and brain lesions, making surgical treatment difficult (Buckmiller et al., 2010).

Both Tie2/Ang-2 and VEGF/KDR receptor-ligand system are involved in normal and pathogenic angiogenesis (Suri et al., 1996). Tie2/Ang-2 system is a signal pathway of angiopoiesis including angiopoietin and its receptor Tie2. This signal pathway plays an important role in the process of angiogenesis in embryonic period and adult stage and appears to be critical for endothelial cell-smooth muscle cell communication in venous morphogenesis. Vikkula et al. (1996) identified a missense arg849-to-trp mutation (rs80338908 in exon 15) in the Tie2 receptor which was shown with the affected phenotype in two families with inherited VMs. The mutation resulted in increased phosphorylation activity of Tie2 and therefore represents an activating mutation. Limaye et al. (2009) identified 8 somatic Tie2 mutations in lesions from 28 of 57 individuals (49.1%) with sporadic venous malformations. The most common one, L914F (rs80338909 in exon 17), accounts for 85% of lesions, and has not been observed as an inherited mutation, which resulted in ligand-independent Tie2 hyperphosphorylation in vitro (Limaye et al., 2009; Wouters et al., 2009; Ye et al., 2011).

KDR signaling is considered crucial to vascular formation. ANTXR1 and KDR play an important role in KDR signaling, the mutations identified in ANTXR1 or KDR can prevent VEGF from activating KDR and its downstream targets. KDR is critical for promoting angiogenesis, which can prevent VEGF from binding, receptor phosphorylation, and subsequent signaling steps (Basu et al., 2010; Fang et al., 2009; Jinnin et al., 2008). ANTXR1 is a tumor-specific endothelial marker highly expressed in tumor endothelial cells but not in normal endothelial cells (St. Croix et al., 2000).

On the basis of the above evidence, we performed the retrospective case–control association study to further investigate whether polymorphisms in genes of VEGF/KDR and Tie2/Ang-2 pathway are associated with VMs in Chinese Han population.

Abbreviations: VM, vascular malformation; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth receptor-2; TEK, tyrosine kinase, endothelial; TEM8, tumor endothelium marker 8; MAF, minor allele frequency; CI, confidence interval; HWE, Hardy–Weinberg equilibrium; OR, odds ration.

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http://dx.doi.org/10.1016/j.gene.2013.02.053

2. Methods

2.1. Subjects

From January 2008 to March 2010, a total of 103 patients with VMs, and 142 unrelated healthy control individuals, were recruited from the Plastic surgery department of the Changhai hospital. A total of 54 patients were male and 49 patients were female, aged from 2 years to 86 years and a mean age of 31.5 years. All of the patients were diagnosed by clinical manifestation, histologic characteristics, and imaging examination. Eighty-two patients suffered from VMs, and twenty-one patients suffered from mixed malformation (venous and capillary malformation). The location of the diseases were extremities (48patients, 46%), face (31 patients, 30%), truck (11 patients, 11%), neck (8 patients, 8%), glutea (2 patients, 2%), scalp (2 patient, 2%), and perineum (2 patients, 2%). A total of 67 lesions were limited in cutaneous and soft tissue, and other 36 lesions also involved muscle (Table 1). Controls were identified as having no signs of vascular anomalies and were recruited from the same hospital (Changhai hospital, Shanghai). Controls had mean ages of 34.1 years. All subjects were Han Chinese in origin. The study complied with the guidelines of our local Medical Ethical Committee and all participants recruited in this study provided written informed consents.

2.2. Genotyping

DNA was extracted from whole blood samples by using the DNA Blood Maxi Kit (Qiagen). Genotyping was performed without knowledge of the clinical status of the subjects.

The sequences of the PCR primers and cycling conditions are given in Supplementary Table 1. PCR amplification was carried out in a 20ul reaction mixture containing 10 ng of DNA, 10 pmol of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTP and 0.25 U Taq DNA polymerase (Sigma, St. Louis, MO, USA). The PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, Foster city, CA). The sequences were analyzed in an ABI PRISM model 3100 DNA Sequencer (PE Applied Biosystems, Perkin-Elmer) to determine the genotypes of variation at the same position on both forward and reverse sequences. Any differences were resolved by regenotyping samples (with overall rate <0.05%).

2.3. Single nucleotide polymorphism selection

Using HapMap Project Build 36, we identified the SNPs in each gene with a minimum allele frequency (MAF) of 10% and captured 6 SNPs in three genes in all. We also choose two additional SNPs (rs80338908 and rs80338909) previously associated with VMs (Limaye et al., 2009; Ye et al., 2011).

2.4. Statistics

Statistics were performed with SPSS (version 13.0). Comparisons of the genotypic or allele frequencies between cases and controls were carried out using the X^2 test. The odds ratio (OR) and their 95% confidence intervals were estimated for the effects of alleles. Linkage disequilibrium statistics were computed using D' and r^2 on Haploview3.32 (Barrett et al., 2005). Haplotype distribution was estimated using the program UNPHASED (Dudbridge, 2003). The comparison of allele and genotype frequencies of each polymorphism between case and control groups was carried out on the online software SHEsis (Table 2) (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He, 2005). For all statistical analyses, $p < 0.05$ was considered statistically significant. Power analysis of our sample was performed using the G*Power 3 program (Faul et al., 2007).

Table 1
Clinical characteristic of vascular malformation.

	Gender		Location						Extent	
	Male	Female	E	FN	T	G	S	P	Cutaneous and soft tissue	Muscle involved
Venous malformation	41	41	34	32	10	2	2	2	56	26
Mixed malformation	13	8	14	6	1	0	0	0	11	10

E: extremities, FN: face and neck, T: truck, G: glutea, S: scalp, P: perineum.

2.5. Prediction of SNP effects

The possible SNP effects on mRNA splicing were predicted using the Web-based prediction software ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) (Smith et al., 2006) and the splice prediction program of the BDGP (http://www.fruitfly.org/seq_tools/promoter.html) (Reese, 2001).

3. Results

To examine the association between VMs and polymorphisms in genes of Tie2/Ang-2 and VEGF/KDR pathway, we identified 4 Tie2 SNPs, 2 KDR SNPs and 2 ANTXR1 SNPs. A total of 103 Han Chinese patients with VMs and 142 ethnically-matched controls were genotyped for this study. All analyzed SNPs conformed to Hardy–Weinberg equilibrium in both the case and control populations. The data for genotypic and allelic frequencies are shown in Table 2. As shown in Table 2, allele distributions did not differ significantly between cases and controls for 2 VEGFA SNPs and 2 ANTXR1 SNPs.

In this study, power analysis showed that the statistical power of our sample to detect a significant association ($p < 0.05$) was 95.1% in genotypic comparisons for VMs, respectively when a large effect size ($w = 0.8$) was presumed. This indicates that the sample size in our study was sufficient to achieve a relatively low risk of a type II error (Faul et al., 2007).

We also tested for associations between the VMs and two SNPs in Tie2 (rs80338908 in exon 15 and rs80338909 in exon 17), but we did not detect any germ-line mutations in exon 15 and exon 17 of the Tie2 gene from patients whole blood samples.

Two SNPs (rs639225 and rs638203) are in close linkage disequilibrium ($D' = 0.970$, $r^2 = 0.923$). In this study, we did find a significant association between the synonymous SNP rs639225 and VMs ($p = 0.018$, odds ratio = 0.642, 95% CI = [0.445–0.926]). The mutant "G" allele of SNP rs639225 was analyzed by ESEfinder; the data indicate that G allele of SNP rs639225 can affect splicing regulation by altering ESE motifs. ESEfinder analysis results shows that the mutant "G" result in the loss of SRp40 motif (2.71) and the gain of another SRp40 motif (4.08) (Fig. 1). We also use a free online utility termed BDGP to predicted aberrant splice site. We found that the SNP rs638203, which located 17 nucleotides downstream of the 3′ splice sites, introduce a new splice site at high efficiency (0.80).

4. Discussion

We analyzed rs638203, rs639225, rs80338908, rs80338909 in Tie2, rs2305949, rs1870377 in KDR and rs79337921, rs34590960 in ANTXR1.

This study is the first to investigate the association of polymorphisms in genes of VEGF/KDR with VMs. Although both KDR and ANTXR1 have been implicated in the pathophysiology of diseases, e.g. infantile hemangioma (Jinnin et al., 2008), we found minimal evidence that polymorphisms in KDR and ANTXR1 contribute significantly to risk of VMs. We only study 4 SNPs in KDR and ANTXR1; it is needed for further investigating the association between the polymorphisms in genes of VEGF/KDR and VMs. Since this association