Association Between Functional Haplotypes of Vascular Endothelial Growth Factor and Renal Complications in Henoch-Schönlein Purpura

BLANCA RUEDA, CRISTINA PEREZ-ARMENGOL, SANDRA LOPEZ-LOPEZ, CARLOS GARCIA-PORRUA, JAVIER MARTÍN, and MIGUEL A. GONZALEZ-GAY

ABSTRACT. ***Objective.*** High expression of circulating vascular endothelial growth factor (VEGF) has been reported in patients with Henoch-Schönlein purpura (HSP). We investigated the role of –1154 G→A (rs1570360) and –634 G→C (rs2010963) *VEGF* gene functional variants in the susceptibility to HSP, to identify associations with severe systemic complications of HSP, in particular with renal complications.

Methods. Fifty-seven patients from the Lugo region of Northwest Spain with primary cutaneous vasculitis classified as HSP according to proposed criteria were studied. All patients were required to have had at least 2 years' followup. Patients and ethnically matched controls (n = 226) were genotyped for the *VEGF* –1154 G→A and –634 G→C polymorphisms using real-time PCR technology based on TaqMan 5' allelic discrimination assay.

Results. No significant differences in the allele or genotype frequencies for the 2 *VEGF* polymorphisms were observed between HSP patients and controls. However, the high VEGF producer *VEGF* –1154 G allele was increased in HSP patients with nephritis compared with healthy controls (p = 0.02, OR 2.13, 95% CI 1.11-4.08; p_c = 0.04). Similarly, the high VEGF producer *VEGF* –634 C allele was increased in patients with nephritis compared to controls (p = 0.04, OR 1.66, 95% CI 1.01-2.73; p_c = 0.08). The –1154G/–634C haplotype was associated with susceptibility to nephritis (p = 0.03, OR 1.71, 95% CI 1.01-2.89). A protective effect against nephritis was observed for the –1154A/–634G *VEGF* promoter haplotype (p = 0.02, OR 0.49, 95% CI 0.30-0.95).

Conclusion. Our results suggest a potential implication of the *VEGF* –1154 G→A and –634 G→C polymorphisms in the development of nephritis in patients with HSP. (J Rheumatol 2006;33:69–73)

Key Indexing Terms:
HENOCH-SCHÖNLEIN PURPURA DISEASE SUSCEPTIBILITY RENAL INVOLVEMENT
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Henoch-Schönlein purpura (HSP) is the most common primary small blood vessel leukocytoclastic vasculitis in children and a rare condition in adults[1]. Palpable purpura and joint and gastrointestinal (GI) manifestations are typical of this vasculitis[2]. Renal manifestations constitute the most feared complications, and longterm morbidity and mortality in HSP are mainly due to renal involvement[3].

Well documented reports of families of first-degree relatives with HSP support a genetic component in the pathogenesis of HSP[1,3]. Susceptibility to HSP and associated clinical heterogeneity in HSP may be conferred by a number of genetic loci. Studies in Northwestern Spain have shown that different genes may influence the phenotype and the outcome of this condition[4-9].

Vascular endothelial growth factor (VEGF) is a heparin-binding growth factor mainly produced by activated monocytes/macrophages and T cells, specific for vascular endothelial cells, which is able to induce angiogenesis *in vivo*[10]. VEGF stimulates functional changes in endothelial cells and enhances endothelial cell proliferation, angiogenesis, microvascular permeability to fluids and plasma proteins, and monocyte chemotaxis. In addition, it activates interstitial collagenase production and von Willebrand factor release and enhances procoagulant activity[10-14].

As with other systemic vasculitides[15,16], high expression of circulating VEGF has been reported in patients with leukocytoclastic vasculitis[17]. Interestingly, Topaloglu, *et al* found a significant increase in the plasma concentrations of VEGF in 22 children with HSP in the acute phase of the disease[18]. However, plasma VEGF levels failed to show any

From the Instituto de Parasitología y Biomedicina López Neyra, CSIC, Granada; and the Division of Rheumatology, Hospital Xeral-Calde, Lugo, Spain.

B. Rueda, PhD; C. Perez-Armengol, PhD; J. Martin, MD, PhD, Instituto de Parasitologia y Biomedicina Lopez-Neyra, CSIC; S. Lopez-Lopez, MD; C. Garcia-Porrua, MD; M.A. Gonzalez-Gay, MD, PhD, Division of Rheumatology, Hospital Xeral-Calde.

Dr. Gonzalez-Gay and Dr. Martin share senior authorship in this study.

Address reprint requests to Dr. M.A. Gonzalez-Gay, Rheumatology Division, Hospital Xeral-Calde, c) Dr. Ochoa s/n, 27004, Lugo, Spain. E-mail:miguelaggay@hotmail.com

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difference between the patients with and those without renal manifestations as well as GI and joint involvement[18].

Several polymorphisms have been described within the *VEGF* 5'UTR region, which is known to regulate VEGF expression at the posttranscriptional level[19,20]. Two single nucleotide polymorphisms (SNP) located within the *VEGF* promoter region, the –1154 G→A (rs1570360) and the –634 G→C (rs2010963), were found to be related with variations in circulating VEGF levels and *VEGF* gene expression[21]. Additionally, the –634 G→C SNP was found to affect the activity of an internal ribosomal entry site B (IRES-B) involved in *VEGF* translation[21]. A recent study in an Italian population showed that the *VEGF* –634 C allele was significantly increased in patients with giant cell arteritis (GCA) compared with matched controls[22].

Taking these considerations into account, we assessed the role of *VEGF* –1154 G→A and –634 G→C functional variants in susceptibility to and severity of HSP.

MATERIALS AND METHODS

Patients and controls. Patients with primary cutaneous vasculitis recruited from the Divisions of Pediatrics and Rheumatology of the Hospital Xeral-Calde (Lugo, Spain) who fulfilled the 1990 American College of Rheumatology criteria for hypersensitivity vasculitis or HSP[23,24] were differentiated using the criteria proposed by Michel, *et al*[25]. They were classified as having HSP if they fulfilled at least 3 of the following 6 criteria: (1) palpable purpura, (2) bowel angina, (3) GI bleeding, (4) macroscopic or microscopic hematuria, (5) age at disease onset ≤ 20 years, and (6) no history of medication taken before the onset of the disease. Patients who fulfilled fewer than 3 criteria were excluded.

As described[4], in adults a skin biopsy showing leukocytoclastic vasculitis was always required. In children, a diagnosis of cutaneous vasculitis was considered if they had typical nonthrombocytopenic symmetric palpable purpura involving the lower extremities, and other conditions such as connective tissue diseases and infections had been excluded. For the purpose of examining the outcome of HSP, only patients with at least 2 years' followup were included in our study.

The main epidemiological and clinical data of the 57 patients with HSP are summarized in Table 1. Of note, although hematuria with or without proteinuria was observed in 37 of 57 patients, at last followup (minimum 2, median 8 yrs) only 11 of the 57 patients had persistent renal involvement (renal sequelae), mainly hematuria, without renal insufficiency.

Ethnically matched controls (n = 226) were also recruited from the Lugo region.

All patients and controls gave written informed consent. We obtained approval for the study from the local ethical committee.

VEGF genotyping. DNA from patients and controls was obtained from peripheral blood using standard methods. Samples were genotyped for –1154 G→A and –634 G→C variants using a TaqMan 5' allelic discrimination assay. The –1154 G→A polymorphism was genotyped using a custom TaqMan SNP genotyping assay method (Applied Biosystems, Foster City, CA, USA). The primers sequences were 5'- TGG GCG TCC GCA GAG (forward) and 5'-CCG CTA CCA GCC GAC TTT TAA (reverse), and the TaqMan MGB probe sequences were 5'-CCT CAG CCC TTC CAC AC and 5'-CTC AGC CCC TCC ACA C; probes were labelled with VIC and FAM fluorescent dyes, respectively. A TaqMan SNP genotyping assay was used for the –634 G→C polymorphisms (number C8311614, Applied Biosystems).

Polymerase chain reaction (PCR) was carried out in a total reaction volume of 12 µl with the following amplification protocol: denaturation at 95°C for 10 min, followed by 55 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Post-PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on an ABI Prism 7000 sequence detection system using SDS 1.1 software for allelic discrimination (Applied Biosystems). We confirmed the assigned genotyping determined by the software using a PCR-restriction fragment length polymorphism of representative samples from each genotype as described[20], and additionally by sequencing these representative samples.

Table 1. Main features of 57 patients with primary cutaneous vasculitis that fulfilled criteria for Henoch-Schönlein purpura.

	N (%)
Children (age < 21 yrs)/adults	45/12
Male/female	28/29
Age at disease onset, yrs	
Median	7
Range	2–62
Duration of followup, yrs, median	8
History of upper respiratory tract infection* (%)	35 (61.4)
History of drug use* (%)	17 (29.8)
Palpable purpura and/or maculopapular rash (%)	57 (100)
Arthralgia and/or arthritis (%)	39 (68.4)
Gastrointestinal manifestations (%)	46 (80.7)
Bleeding	23 (40.4)
Bowel angina	43 (75.4)
Renal manifestations (%)	
Hematuria (≥ 10 red blood cells/high power field)	37 (64.9)
Proteinuria (> 500 mg/24 h)	19 (33.3)
Nephrotic syndrome	7 (12.3)
Renal insufficiency	2 (3.5)
Renal sequelae (persistent renal involvement)** (%)	11 (19.3)

* Within one week before onset of vasculitis. ** At last followup (minimum 2 yrs).

Statistical analysis. Strength of association between HSP patient groups and controls and alleles or genotypes of the VEGF polymorphisms was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by chi-square or Fisher exact analysis. Statistical significance was defined as $p \leq 0.05$. P values were corrected (p_c) by the number of comparisons. Calculations were performed with the Stata V6 statistical package. For the haplotype analysis, pairwise linkage disequilibrium measures were investigated and haplotypes constructed using the expectation-maximization (EM) algorithm in Unphased software. We estimated the pairwise-disequilibrium coefficient D', which detects the probability of observing 2 marker alleles on the same haplotype or observing them independently in the population, with D' = 1 complete linkage disequilibrium and D' = 0 total independence[26].

RESULTS

Table 2 shows the genotypic and allelic frequencies of *VEGF* –1154 G→A and –634 G→C promoter genetic variants in HSP patients and controls.

The control population was found to be in Hardy-Weinberg equilibrium. Allelic and genotypic frequencies of both *VEGF* –1154 G→A and –634 G→C polymorphisms observed in our population were in concordance with those published for other Caucasian populations[21].

The overall distribution of *VEGF* –1154 G→A or –634 G→C genetic variants did not show any statistically signif-

Table 2. Genotypic and allelic frequencies of *VEGF* –1154 G→ A and –634 G→ C promoter polymorphisms among HSP patients and controls.

VEGF Promoter Polymorphisms	HSP Patients, n = 57 (%)	Controls, n = 226 (%)
–1154 G→ A		
Genotype*		
G/G	38 (66.7)	118 (52.2)
G/A	14 (24.6)	84 (32.2)
A/A	5 (8.8)	24 (10.6)
Allele		
G	90 (78.9)	320 (70.8)
A	24 (21.1)	132 (29.2)
–634 G→ C		
Genotype		
G/G	18 (31.6)	101 (44.7)
G/C	27 (47.4)	86 (38.1)
C/C	12 (21.1)	39 (17.2)
Allele		
G	63 (55.3)	288 (63.7)
C	51 (44.7)	164 (36.3)

* GG versus GA + AA *VEGF* –1154 genotype in HSP patients compared with controls (p = 0.05, OR 1.33, 95% CI 1.00–3.37).

icant deviation between HSP patients and controls considering genotype or allele frequencies (Table 2). In this regard, although *VEGF* –1154 G/G genotype was increased in HSP patients compared to ethnically matched controls (p = 0.05, OR 1.33, 95% CI 1.00-3.37; Table 2), this difference was not significant when Bonferroni correction was applied.

To determine whether *VEGF* promoter polymorphisms were implicated in disease severity, we stratified HSP patients according to the presence of nephritis, renal sequelae at last followup, and severe GI manifestations. With this procedure we found no significant differences among the subgroups of HSP patients (Table 3). However, when patients with nephritis were compared with healthy controls some differences were observed. In this regard, the high VEGF producer *VEGF* –1154 G allele[21] was increased in HSP patients with nephritis compared with healthy controls (p = 0.02, OR 2.13, 95% CI 1.11-4.08; p_c = 0.04; Table 3). In addition, all the patients with renal sequelae carried this high VEGF producer *VEGF* –1154 G allele. However, due to the small number of patients with renal sequelae (n = 11), the difference with respect to controls was not significant when a Bonferroni correction was applied (p = 0.04, OR 4.13, 95% CI 0.95-17.90; p_c = 0.08; Table 3). Similarly, although the high VEGF producer *VEGF* –634 C allele[21] was also increased in patients with nephritis compared to controls, p values were not significant when they were corrected by the number of comparisons (p = 0.04, OR 1.66, 95% CI 1.01-2.73; p_c = 0.08; Table 3).

We also estimated haplotypes for the *VEGF* –1154 G→A and –634 G→C *VEGF* promoter variants using the EM algorithm. Pairwise maximum-likelihood analysis of linkage disequilibrium (D') showed complete linkage disequilibrium between the 2 variants (Table 4). Among the 4 possible haplotypic combinations, 3 common haplotypes were

Table 3. Genotypic and allelic frequencies of *VEGF* –1154 G → A and –634 G → C promoter polymorphisms according to HSP patients' clinical manifestations.

VEGF Promoter Polymorphisms	HSP and Nephritis,		HSP and Renal Sequelae,		HSP and GI Manifestations*,		Controls, n = 226 (%)
	Yes n = 37 (%)	No n = 20 (%)	Yes n = 11 (%)	No n = 46 (%)	Yes n = 46 (%)	No n = 11 (%)	
–1154 G→ A							
Genotype							
G/G	27 (73.0)	11 (55.0)	9 (81.8)	29 (63.0)	31 (67.4)	7 (63.9)	118 (52.2)
G/A	8 (21.6)	6 (30.0)	2 (18.2)	12 (26.1)	12 (26.1)	2 (18.2)	84 (37.2)
A/A	2 (5.4)	3 (15.0)	0 (0.0)	5 (10.9)	3 (6.5)	2 (18.2)	24 (10.6)
Allele							
G	62 (83.8)[a]	28 (70.0)	20 (90.9)[b]	70 (76.1)	74 (80.4)	16 (72.7)	320 (70.8)[a,b]
A	12 (16.2)	12 (30.0)	2 (9.1)	22 (23.9)	18 (19.6)	6 (27.3)	132 (29.2)
–634 G→ C							
Genotype							
G/G	10 (27.0)	8 (40.0)	3 (27.3)	15 (32.6)	15 (32.6)	3 (27.3)	101 (44.7)
G/C	18 (48.7)	9 (45.0)	7 (63.6)	20 (43.5)	23 (50.0)	4 (36.4)	86 (38.1)
C/C	9 (24.3)	3 (15.0)	1 (9.1)	11 (23.9)	8 (17.4)	4 (36.4)	39 (17.2)
Allele							
G	38 (51.4)	25 (62.5)	13 (59.1)	50 (54.3)	53 (57.6)	10 (45.5)	288 (63.7)
C	36 (48.6)[c]	15 (37.5)	9 (40.9)	42 (45.7)	39 (42.4)	12 (54.5)	164 (36.3)[c]

* Gastrointestinal bleeding and/or bowel angina. [a] *VEGF* –1154 G allele was increased in HSP patients with nephritis compared with healthy controls (p = 0.02, OR 2.13, 95% CI 1.11–4.08; p_c = 0.04). [b] *VEGF* –1154 G allele was increased in HSP patients with renal sequelae compared with healthy controls (p = 0.04, OR 4.13, 95% CI 0.95–17.90; p_c = 0.08). [c] *VEGF* –634 C allele was increased in HSP patients with nephritis compared with healthy controls (p = 0.04, OR 1.66, 95% CI 1.01–2.73; p_c = 0.08).

Table 4. Distribution of VEGF promoter haplotypes defined by –1154 G→ A and –634 G → C polymorphisms in HSP patients and controls.

VEGF Promoter Haplotypes	HSP Patients, N = 57 (%)	HSP and Nephritis, Yes[a] n = 37 (%)	HSP and Nephritis, No n = 20 (%)	Controls[a], N = 226 (%)
–1154G/–634C	51 (44.7)	36 (48.7)[b]	15 (37.5)	159 (35.3)[b]
–1154A/–634G	24 (21.1)	12 (16.2)[c]	12 (30)	127 (28.2)[c]
–1154G/–634G	39 (34.2)	26 (35.1)	13 (32.5)	160 (35.6)
D´	1	1	1	0.98

[a] The overall distribution of *VEGF* promoter haplotypes between patients with nephritis and controls showed a statistically significant difference (p = 0.03). [b] –1154G/–634C *VEGF* promoter haplotype was increased in HSP patients with nephritis compared with healthy controls (p = 0.03, OR 1.71, 95% CI 1.01–2.89). [c] –1154A/–634G *VEGF* promoter haplotype was decreased in HSP patients with nephritis compared with healthy controls (p = 0.02, OR 0.49, 95% CI 0.30–0.95).

observed in our study population (–1154G/–634C, –1154A/–634G, and –1154G/–634G). No statistically significant skewing was observed for the distribution of *VEGF* promoter haplotypes between HSP patients and controls. Interestingly, the –1154G/–634C haplotype related with higher serum concentration of VEGF was significantly increased in HSP patients with nephritis (p = 0.03, OR 1.71, 95% CI 1.01-2.89) compared with controls (Table 4). Further, the low VEGF producer haplotype –1154A/–634G showed the opposite tendency, being significantly increased among healthy controls (p = 0.02, OR 0.49, 95% CI 0.30-0.95).

DISCUSSION

Our study is the first to investigate the potential implications of 2 functional polymorphisms in the *VEGF* 5'UTR region for disease susceptibility and severity of patients with primary cutaneous vasculitis. Our results suggest a potential role of the *VEGF* –1154 G→A and –634 G→C polymorphisms in the development of nephritis in patients with cutaneous vasculitis who met classification criteria for HSP.

We also assessed the implication of functional *VEGF* genetic variants in susceptibility to and clinical complications of biopsy-proven GCA. Our results did not provide evidence implicating *VEGF* –1154 G→A or –634 G→C promoter polymorphisms in predisposition to GCA. However, an interesting finding was the association of *VEGF* –634 G→C polymorphism with specific clinical manifestations of GCA[27]. In this regard, the low VEGF producer G allele was significantly overrepresented in GCA patients with ischemic complications, and additionally, a higher risk of developing severe ischemic complications was observed for –634 GG homozygous individuals[27]. Thus, in GCA, high production of VEGF was associated with protection against the development of severe ischemic complications, probably mediated by neoangiogenesis mechanisms. However, the pathogenesis of HSP, an IgA-mediated vasculitis involving small blood vessels, and that of GCA, affecting preferentially large and middle-sized vessels, are different.

In the kidney, expression of VEGF is most prominent in glomerular podocytes and in tubular epithelial cells, while VEGF receptors are found on preglomerular, glomerular, and peritubular endothelial cells. VEGF and its receptors are upregulated in animal models and humans with type 1 and type 2 diabetes[28]. Inhibition of VEGF has beneficial effects on diabetes-induced functional and structural alterations, suggesting a deleterious role for VEGF in the pathophysiology of diabetic nephropathy[28]. Of note, the 2 genetic variants associated with nephritis in our patients with HSP have been found to be high VEGF producers[21]. We confirmed the tendency observed for the implication of VEGF alleles in severity of HSP at the haplotype level. The higher VEGF producer haplotype –1154G/–634C was significantly increased in HSP patients with nephritis, and thus it might be implicated in the pathogenesis of the nephritis in patients with this vasculitis. As in the case of diabetic nephropathy, it might be interesting to assess the inhibition of *VEGF* as a useful therapeutic tool in the prevention of nephritis in patients with HSP.

In HSP, genetic factors are thought to determine immunological and inflammatory responses to unknown antigens; we have previously described the involvement of different inflammatory mediators in HSP. A biallelic (–511 C/T) polymorphism in the interleukin 1 (IL-1) gene was directly implicated in the severity and outcome, but not the susceptibility, of cutaneous vasculitis in unselected patients[9]. In addition, an association between an IL-1 receptor antagonist variable number of tandem-repeat gene polymorphisms and the development of severe renal manifestations and renal sequelae in HSP has been described[7]. These observations, together with our findings regarding VEGF polymorphisms, support the hypothesis discussed in previous studies[4-9,29] about the potential role of different genetic factors influencing susceptibility to HSP and its severity. It is known that VEGF production can be stimulated by differ-

ent proinflammatory cytokines[14]. Therefore, it could be speculated that these genetic polymorphisms exert interdependent effects in predisposition to and severity of HSP. Another possibility may be that each genetic variant has an individual effect and may contribute independently with a low effect on genetic risk for HSP.

It will be interesting to search for novel potential candidate genes in HSP susceptibility and severity. A wide number of inflammatory mediators seem to be implicated in the regulation of new vessel formation and vasculitis. Various mediators implicated in vasculogenesis, for instance, HIF-1 and VEGF receptors and beta$_2$-integrins, could be proposed as candidate genes in predisposition to HSP[30-32].

Our observations may contribute to existing knowledge of stratification of patients with primary cutaneous vasculitis. Studies in other populations, and in particular in larger numbers of patients, are required to confirm the association between *VEGF* gene polymorphisms and development of nephritis in patients with HSP.

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