Implication of *VEGFR2* in systemic lupus erythematosus: a combined genetic and structural biological approach

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Abstract

Objectives

VEGFR2 *gene polymorphisms have already been correlated with vascular diseases such as coronary heart disease (CHD) and may influence endothelial integrity, repair and function. In view of the premature atherosclerosis observed in SLE, we sought to clarify the structural/functional consequences of two common single nucleotide polymorphisms (SNPs) of* VEGFR2 *in SLE and determine whether they are associated with risk of SLE by influencing endothelial cells.*

Methods

Three-dimensional (3D) homology modelling was applied for the localisation of the V297I and the Q472H polymorphisms. Genotyping of the V297I (rs2305948) and Q472H (rs1870377) SNPs was done through Taqman technology in 250 SLE patients and 241 healthy controls from a Greek population (Cretan). The replication sample set for the rs1870377 SNP consisted of 253, 184 and 77 patients with SLE and 301, 118 and 11 ethnically-matched controls of African-American, European-American and Hispanic-American origin, respectively.

Results

Modelling revealed that the V297I polymorphism may affect the efficiency of trans-autophosphorylation and cell signalling, while Q472H affects homotypic contacts of membrane proximal Ig-like domains. No significant allelic and genotypic association was observed for both the SNPs with risk of SLE.

Conclusion

Although structural data suggest that both VEGFR2 *SNPs may contribute to SLE pathogenesis by impairing VEGF signalling, none of the SNPs analysed was associated with increased susceptibility to SLE. However, they still may be relevant to the vascular damage/atherosclerosis in SLE.*

Key words

gene polymorphisms, systemic lupus erythematosus, association study, three-dimensional (3D) model

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Introduction

Angiogenesis and lymphangiogenesis, the growth of new blood and lymphatic vessels from preexisting ones, are important biological processes during embryonic development, tissue growth, wound healing, and in the pathogenesis of various diseases such as cancer, diabetic retinopathy, psoriasis, arthritis and systemic sclerosis (1-5). An angiogenic mediator which has attracted much attention is Vascular Endothelial Growth Factor (VEGF), which is an endothelial selective growth factor. Vascular endothelial growth factors' action largely restricted to endothelial cells is considered to be the principal angiogenic growth factor that mediates signals for endothelial cell proliferation and viability and induces endothelial cell migration and sprouting, thus contributing to efficient angiogenesis (6). To the best of our knowledge, VEGF is produced by endothelial cells, monocytes and fibroblasts in response to hypoxia, which is the major physiological signal for angiogenesis. However, under certain pathological conditions, VEGF can be produced by tumour cells or activated T-lympocytes inducing inflammatory angiogenesis, known as neo-angiogenesis. It also regulates blood and lymph vessel formation through the activation of three receptor tyrosine kinases, VEGFR-1, -2, and -3. Binding of growth factors to the ectodomain of their transmembrane receptors leads to receptor dimerisation, protein kinase activation, trans-autophosphorylation, and initiation of signalling pathways (7). Indeed, the receptors are activated upon ligand binding to the extracellular receptor domain (ECD), resulting in receptor dimerisation and activation of the intracellular tyrosine kinase domain of the receptor (8).

The association of VEGF with *VEGFR2* (kinase domain receptor/FLK-1) is a critical event in angiogenesis (9). Therefore, ligand binding to the extracellular domain of *VEGFR-2* (10) promotes conformational changes resulting in receptor dimerisation (Fig. 1). Importantly, *VEGFR-2* is responsible for the majority of the angiogenic and permeability-enhancing effects of VEGF (11, 12), while VEGFR-1 regulates VEGF activity in the vascular endothelium by preventing VEGF/*VEGFR-2* binding (11). *VEGFR2* gene is located at the 4q11-13 chromosomal region (13) and the encoded protein is considered to be the principal receptor that mediates VEGF signals in endothelial cells and belongs to the receptor-tyrosine kinases superfamily. The extracellular region of the receptor is composed of 7 Ig-like domains, with the ligand binding region located on the 2nd-3d Ig-like domains. The 4th-7th Ig-like extracellular domains are considered crucial for signal transduction. The *VEGFR2* directly regulates the formation of blood vessels, a process dependent on the function of specialised cell-types – *i.e.* vascular endothelial cells. Signalling by *VEGFR2* represents the major pathway for the transduction of angiogenic signals and, therefore, is a prominent target in the development of angiogenesis inhibitors (14).

Two *VEGFR2* functional gene polymorphisms, V297I (1192 G/A, rs2305948) and Q472H (1719 T/A, rs1870377), have already been correlated with the development of vascular diseases and it has been suggested that these polymorphisms may be important for the ligand binding of *VEGFR2* (15). Although higher serum VEGF levels have been associated with SLE patients as compared to controls (16), to our knowledge, the potential link between the *VEGFR2* gene and systemic lupus erythematosus (SLE) has not been investigated. Assuming that genes involved in vascular endothelium damage may confer risk for different vascular-mediated diseases, we hypothesised that variation of *VEGFR2* gene may change the biological function of the protein and, as a consequence, this genetic factor may influence the endothelial function in patients with SLE. On the basis of this background, this study was designed: a) to construct a three-dimensional (3D) model of the *VEGFR2* regions harbouring the polymorphisms rs2305948 and rs1870377, localise them on this model and explore their putative role on the *VEGFR2* function, and b) to investigate the contribution of these polymorphisms in the development of SLE.

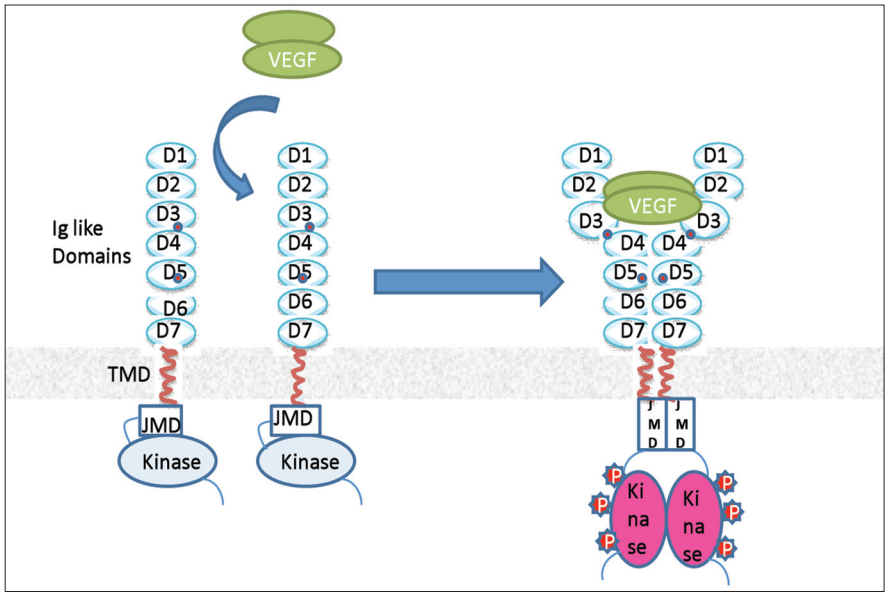

Fig. 1. Schematic diagram of *VEGFR2* activation mechanism. Upon binding of VEGF dimer (green) on two *VEGFR2* receptors dimerisation occurs, leading to D4-D7 homotypic interactions and resulting in kinase activation (in red) and autophosphorylation of tyrosine residues (indicated with red stars and P) required for downstream signalling. D1-D7 are the Ig like domains of *VEGFR2*, TMD and JMD the trans- and juxta-membrane domains, respectively. Mutations V297I on domain D3 and Q472H on domain D5 are indicated with red dots. Figure adapted from Stuttfeld and Ballmer-Hofer (8).

Patients and methods

Study populations

The primary sample set consisted of 250 SLE patients from the genetic homogeneous population of Crete, who are followed at the outpatient clinic of the Department of Rheumatology, University Hospital of Heraklion, Crete. All patients met the 1982 American College of Rheumatology (ACR) revised classification criteria (16). Exclusion criteria were the presence or suspicion of an overlap autoimmune or chronic inflammatory disease, acute or chronic infectious disease, history of cancer or other malignancies and recent surgery or other active pathological conditions (*e.g.* cardiovascular event) accompanied by systemic inflammatory reaction. Two hundred forty one age- and gender-matched healthy individuals were recruited from the Department of Transfusion Medicine. The study was approved by the ethics committee of the University Hospital of Crete. We used also three replication samples, one African-American (AA) consisting of 253 SLE patients and 301 controls, a European-American (EA) consisting of 184 cases and 118 controls and a smaller one, Hispanic-American (HA), consisting of 77 patients with SLE and 11 controls.

Genetic analysis of the VEGFR2 *polymorphisms*

Whole blood was collected in EDTA-containing tubes and genomic DNA was isolated from peripheral blood leucocytes by using the commercial kit PUREGENE (Gentra SYSTEMS, Minnesota, USA). The extracted DNA was stored at -20°C until analysed. The rs2305948 1192 G/A (V297I) and rs1870377 1719 T/A (Q472H) single nucleotide polymorphisms (SNPs) in *VEGFR2* were genotyped via TaqMan 5'allelic discrimination technology, using predesigned SNPs genotyping assays provided by Applied Biosystems (Foster City, California, USA) (TaqMan assay numbers C_22271999_20 and C_11895315_20, respectively). Neither SNP deviated significantly from the expected Hardy-Weinberg proportions (*p*-values for deviation were all >0.18). The SNPs were genotyped with >98% success rate for each SNP (missingness =2%).

Construction of VEGFR2 *domains' three-dimensional (3D) model*

Modelling of the mutation V297I was based on the 3D structure of domains D2 and D3 of *VEGFR2* in complex with VEGF-C (PDB code 2X1W) (17). In order to investigate mutation Q472H, sequence alignment of the D5 domain of *VEGFR2* was performed on the D7 domain of the *VEGFR2* sequence and KIT sequence using TCOFFEE (18), followed by homology modelling using MODELLER (19) on the experimentally determined structures of D2-D3 *VEGFR2* domains (PDB code 2X1W) (17), the D7 *VEGFR2* domain (PDB code 3KVQ) (20) and the KIT ectodomain structure (PDB code 2E9W) (21). The PDB archive used contains information about experimentally-determined structures of proteins, nucleic acids, and complex assemblies. The QUANTA-CHARMm programme was used to check the derived models for folding and packing errors in order to arrive to a refined combined model with optimal atom contacts (22).

Statistical analysis

Statistical analysis was performed with GraphPad Prism statistical program (GraphPad Software, San Diego, CA). In case-control comparisons, only unrelated individuals were used. The x^2 test, with one or two degrees of freedom, was used to examine differences of genotype and allele frequencies between patients and controls. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated according to Rothman test. The Mann-Whitney U-test was used for comparisons among groups with small or unequal sample sizes. Results are expressed as the mean±SEM (Standard Error of the Mean) for quantitative variables unless otherwise indicated and a two-tailed *p*-value less than 0.05 was defined as statistically significant.

To describe the statistical power of the study, we have now calculated the genetic effect that we would have been able to detect with 80% power given the sample size included in our study. We calculated that for rs1870377, a genetic effect which confers an odds ratio of 1.31 or greater would be detectable in meta-analysis with 80% power with an alpha of 0.05, and presuming a fixed effect that is consistent across the different populations. If each population was considered separately, odds ratios detectable using the same parameters

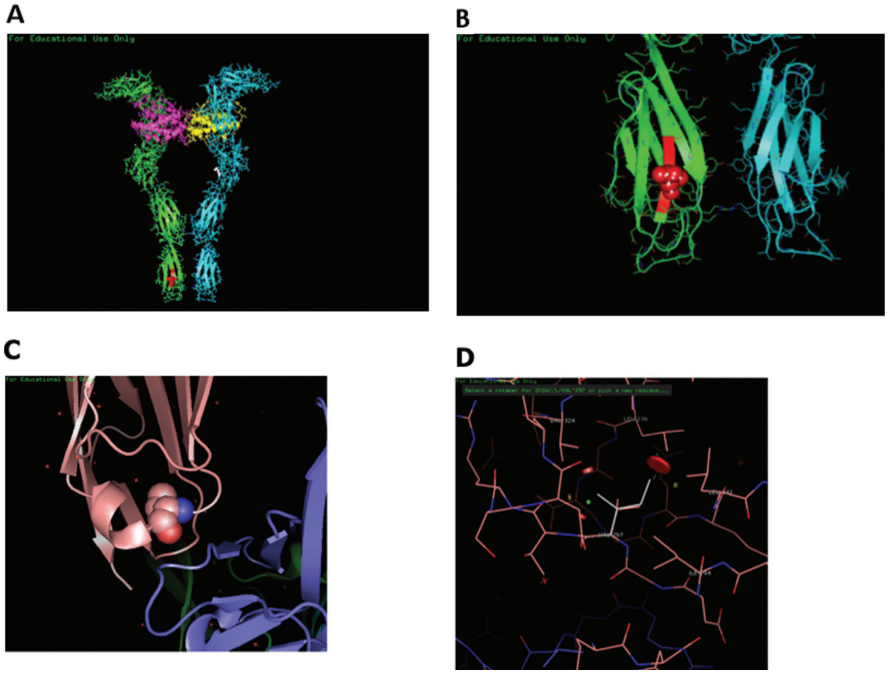

Fig. 2. A) Position n. 472 is located on the surface of the D5 Ig-like domain which shows homology to the D4 KIT domain. **B)** Polymorphism Q472H may affect homotypic contacts of membrane proximal Ig-like domains and thus affect receptor dimerisation like on the D4 and D7 domains of *VEGFR2*. (**A**) Space-filling model indicates approximate position of mutated aminoacid (in red) on a model of the *VEGFR2* dimer. (**B**) Details of the mutation Q472H (in red) on the D5 Ig-like domain ribbon model of the *VEGFR2* dimer. Amino acid position n. 297 is located on the D3 Ig-like domain of the extracellular region of *VEGFR2* on a surface loop (mutation V297I may affect the efficiency of trans-autophosphorylation and cell signalling). Any mutation at this point (shown as Van der Walls spheres in the pink chain [**C**] and white lines [**D**]), especially a hydrophobic one, may affect the conformation of the aminoacid chain loop, inducing changes in the interaction with the D4 domain below (in blue). Red discs indicate bad contacts on mutation of Val to Ile, thus forcing the aminoacid chain to distortion. Position n. 297 is shown in red on the *VEGFR2* truncated dimer model. All pictures were created using Pymol (26).

as above range from 1.54 to 1.78, excluding the Hispanic-American cohort, which contained only 11 controls and did not have any significant independent power. Power calculations were performed using the CATS power calculator by Skol *et al*.

Results

Analysing the structural consequences of Q472H and *V297I* VEGFR2 *polymorphisms*

The rs1870377 SNP causes a non-synonymous amino acid substitution in the coding sequence of *VEGFR2*, thus resulting in a substitution of glutamine (Q) by histidine (H) at position #472 of the protein sequence. Similarly, rs2305948 SNP leads to the substitution of valine (V) by isoleucine (I) at the position n. 297 of the protein chain. Position n. 472 is located on the surface of a β-strand, forming the Ig-like β-barrel of the D5 Ig-like domain of the extracellular *VEGFR2* part (Fig. 2). Mutation Q472H may cause impairment of homotypic contacts of membrane proximal Ig-like domains in the D5-D5 dimer interaction. Modelling revealed that amino acid position n. 297 is located on the D3 Ig-like domain of the extracellular region of *VEGFR2*, involved in ligand (VEGF) binding, on a surface loop in contact with the D4 Ig-like domain, which is responsible for homotypic D4 contacts (20) upon ligand-mediated dimerisation of the VEGF receptors (8). Any mutation at this point (shown as Van der Walls spheres in the pink chain in Figure 2C and in Figure 2D in detail), especially a hydrophobic one such as isoleucine, may affect the conformation of the amino acid chain loop, inducing changes in the interaction with the D4 domain. As a consequence, mutation V297I may affect the efficiency of trans-autophosphorylation and cell signalling. Sequence alignment between the D5 domain and the D7 domain of same receptor and KIT D4, known to form homotypic receptor contacts in *VEGFR2* and KIT dimerisation, respectively, indicate homology between the domains on sequence motifs responsible for mediating homotypic contacts.

The VEGFR2 *rs1870377 SNP is not associated with SLE in four independent (ethnic and racial) populations*

The average age in the Greek cohort was 41.92±12.15 years, and 92% of the subjects were female. The average age in the AA, EA and HA subjects was 43, 47 and 40, respectively, while the percentage of female subjects for these groups was 90.3, 87.6 and 88.3, respectively. Samples were genotyped for the Q472H (rs1870377) SNP in *VEGFR2* located in the fifth extracellular Ig-like domain, in 250 SLE patients and 241 healthy controls via TaqMan 5'-allelic discrimination technology, using predesigned SNPs genotyping assays provided by Applied Biosystems (Foster City, California, USA). Although allele **A** of rs1870377 initially showed a trend toward association in the initial cohort of SLE patients, upon increase of the sample's size there were no significant differences between the cases and controls (p=0.59, OR=1.08, 95%CI 0.81–1.44). Moreover, **A/A** genotype did not appear to be a SLE risk factor (p=0.23, OR=1.24, 95%CI 0.87–1.78). The rs1870377 was found in HWE in the control group (p>0.01). Genotyping accuracy following the Taqman assays method has been found to be greater than 99%.

Consistent with these results, no case-control association was found in replication experiments that involved 3 additional cohorts (Table II). No allelic or genotypic association was observed in either the African-American or the Hispanic-American population analysed, while the same is true regarding the European-American population analysed. This finding is reminiscent of the data collected from the Cretan cohort (Southern European population), although the European-American subjects' group represents a mix of different regions in Europe, and is more frequently of

Table I. Genotypes and allele frequencies of rs1870377 SNP in the *VEGFR2* gene analysed in 250 SLE patients and 241 healthy controls from Greece.

	Genotype			Allele	
	TT	TA	AA	T	A
Cretan cohort					
SLE patients (n=250)	135 (54%)	99 (39.6%)	16 (6.4%)	369 (73.8%)	131 (26.2%)
Healthy controls (n=241)	143 (59.33%)	77 (31.95%)	21 (8.71%)	363 (75.31%)	119 (24.68%)
*OR (95% CI)			1.24 (0.87–1.78)		1.08 (0.81–1.44)
**p-value (d.f. 1)			0.23		0.59

*OR (95%CI): Odds ratio with 95% confidence interval; d.f.: degrees of freedom.
**p-values with d.f. 1 and OR (95%CI) were calculated taking as reference the T/T genotype or the major (T) allele.

Table II. Genotypes and allele frequencies of rs1870377 SNP in the *VEGFR2* gene analysed in three replication groups.

	Genotype			Allele	
	TT	TA	AA	T	A
African-American cohort					
SLE patients (n=253)	217 (85.77%)	31 (12.25%)	5 (1.97%)	465 (92%)	41 (8%)
Healthy controls (n=301)	245 (81%)	52 (17.27%)	4 (1.32%)	542 (90%)	60 (10%)
*OR (95%CI)			0.72 (0.46–1.15)		0.79 (0.53–1.21)
**p-value (d.f. 1)			0.17		0.28
European-American					
SLE patients (n=184)	115 (62.50%)	52 (28.26%)	17 (9.23%)	282 (77%)	86 (23%)
Healthy controls (n=118)	65 (55.08%)	47 (39.83%)	6 (5.08%)	177 (75%)	59 (25%)
*OR (95%CI)			0.74 (0.46–1.18)		0.91 (0.63–1.34)
**p-value (d.f. 1)			0.20		0.65
Hispanic-American					
SLE patients (n=77)	55 (71.42%)	21 (27.27%)	1 (1.29%)	131 (85%)	23 (15%)
Healthy controls (n=11)	6 (54%)	4 (36.36%)	1 (9.09%)	16 (73%)	6 (27%)
*OR (95%CI)			0.48 (0.13–1.74)		0.46 (0.17–1.32)
**p-value (d.f. 1)			0.30		0.21

*OR (95%CI): odds ratio with 95% confidence interval; d.f.: degrees of freedom.
**p-values with d.f. 1 and OR (95%CI) were calculated taking as reference the T/T genotype or the major (T) allele.

North and Western European ancestry. Therefore, it may be assumed that this polymorphism is not mostly relevant for European populations of a specific geographic origin. In addition, the genotypic frequencies of the *VEGFR2* SNP in all these replication groups were in HWE in control groups (p=0.51 in AA, p=0.49 in EA, p=0.48 in HA).

Interestingly, when we examined in a preliminary experiment a putative association between the number of circulating endothelial cells (CECs) and rs1870377 SNP in 59 SLE patients from the Greek cohort, a significant association was found, with the TT genotype bearing higher number of CECs (median number 25) when compared to TA (median number 23, p=0.0144 by unpaired t-test) or AA genotype (median number 13, p=0.0146) (detailed data not shown). However, when we examined the possible influence of this SNP in SLE clinical cardiovascular manifestations (defined as previous myocardial infarction, angiographically confirmed coronary artery disease or peripheral arterial disease and ischaemic stroke) or with subclinical endothelial damage, no association was found.

In a preliminary experiment, a group of 119 SLE patients and 98 controls was genotyped for the V297I (rs2305948) SNP located in the third extracellular Ig-like domain but, given that the allele and genotype frequencies did not differ between cases and controls, no more samples (or populations) were enrolled in the genotyping of this SNP (p=0.8, OR=1.09, 95%CI 0.55–2.15; detailed data not shown).

Discussion

To better understand the putative role of *VEGFR2* in SLE, we performed the present structural biological and genetic study. The structural data presented in the current study suggest that both the polymorphisms may cause impairment in cell signalling, thus contributing possibly to SLE pathogenesis. Indeed, our structural analysis provides insights into receptor/ligand interactions, which are essential for the understanding of receptor/ligand specificity, and explain previously published data. The V297I polymorphism may play an important role in D3-D4 association, thus affecting the efficiency of trans-autophosphorylation, kinase activation and cell signalling mechanism. The Q472H polymorphism may be affecting homotypic contacts of membrane proximal Ig-like domains, ensuring that the transmembrane and cytoplasmic regions of two receptor monomers are brought to a close proximity and correct orientation. It is possible that these genetic variations may result in altered

VEGF binding to *VEGFR2* or impaired signal transmission through the receptor. Notably, it was previously suggested that both SNPs resulted in slight but significant increase in the VEGF binding efficiency to *VEGFR2*. Therefore, it could be speculated that impairment in *VEGFR2* function may be correlated with vascular dysfunction, including endothelial cell damage, impaired endothelial cell survival, decreased antiapoptotic effects of VEGF, and abnormal vascular repair (15).

In this study, by performing a case-control association study, we attempted to reveal for the first time an association of two functional *VEGFR2* SNPs in susceptibility to SLE in a Greek population. However, neither the rs2305948 (V297I) nor the rs1870377 (Q472H) *VEGFR2* SNP polymorphism were found to be associated with increased susceptibility to SLE. Notably, the genotype frequencies of the 2 SNPs from the International HapMap data (23) were very similar to ours. *VEGFR2* gene locus has not been identified in GWAS for SLE susceptibility, a finding that is confirmed by the present "candidate-gene approach" case-control study.

Angiogenesis plays both beneficial and detrimental roles in normal and disease processes. Promotion of the angiogenic response has been demonstrated to be beneficial in treating ischaemic conditions, such as myocardial ischaemia/infarction (25). Of note, *VEGFR2* plays a crucial role in mediating angiogenic endothelial cell responses via the VEGF pathway and inhibitors of angiogenesis targeting *VEGFR2* are in clinical use. Using the structure regions of *VEGFR2* described here in correlation to the polymorphic sites studied, it can be assumed that they can serve as binding sites (of receptor) to block antibodies, which can be used in clinical trials as inhibitors of angiogenesis by targeting the VEGF receptors. Targeting VEGF receptors represented one approach that has enjoyed some therapeutic success for the treatment of metastatic renal cell carcinoma and advanced hepatocellular carcinoma, respectively (14, 26). Preliminary results showed that the rs1870377 *VEGFR2* SNP was associated with higher CECs numbers, thus possibly indicating that this polymorphism may be correlated with increased endothelial damage, an observation that has to be verified in a larger number of patients.

In summary, this study is the first to investigate the association of *VEGFR2* polymorphisms with SLE in different cohorts to date. Apart from the important role of the SNPs investigated in the VEGF/*VEGFR2* binding efficiency, and although their established implication in the pathophysiology of diseases related to disturbance of the endothelial function, we found no evidence that these polymorphisms contribute significantly either to risk or to severity of SLE. However, it is still possible that other genetic variations in or near the *VEGFR2* locus may play a role in SLE.

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