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Influence of nitric oxide synthase gene polymorphisms on the risk of cardiovascular events in rheumatoid arthritis

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ABSTRACT

Objective. *Complex interactions between environmental and genetic determinants in both the host immune system and the vasculature may operate modifying the vascular risk in rheumatoid arthritis (RA). An increased incidence of cardiovascular (CV) events in RA patients carrying HLA-DRB1 shared epitope alleles, in particular HLA-DRB1*0404, has recently been found. In the present study we have assessed the potential contribution of inducible and endothelial nitric oxide synthase (NOS2A and NOS3) gene polymorphisms to CV events in a cohort of patients with rheumatoid arthritis (RA). Also, interactions between NOS2A or NOS3 gene polymorphisms and HLA-DRB1 alleles for the risk of developing CV events were assessed.*

Patients and methods. *One hundred and eighty-two consecutive patients fulfilling the 1987 American College of Rheumatology classification criteria for RA seen at the Rheumatology outpatient clinic of Hospital Xeral Calde, Lugo, Northwest Spain, between March and September 1996 were included. Patients were genotyped by PCR based techniques for a multiallelic $(CCTTT)_n$ repeat in the promoter region of the NOS2A gene and for a T/C polymorphism at position -786 in the promoter region and a polymorphism in exon 7 (298Glu/Asp or 5557G/T) of the NOS3 gene. They were prospectively followed and clinical records were examined until patient's death or September 1, 2005. At the end of the study 39 (21%) patients had experienced CV events.*

Results. *No significant differences in allele or genotype frequencies for the NOS2A promoter CCTTT repeat microsatellite and NOS3 gene polymorphisms between RA patients with or without CV events were found. However, an increased frequency of CV events was observed in RA patients who carried the HLA-DRB1*0404 allele and were homozygous for the NOS3 (-786) TT genotype (OR: 9.06 [95% CI: 1.29-63.37]; p= 0.03) or for the presence of long NOS2A alleles (OR: 11.7 [95% CI: 1.53-88.4]); p= 0.02).*

Conclusions. *Our results show that NOS2A or NOS3 gene polymorphisms do not infer a direct risk for CV events in RA. However, some interactions between NOS gene polymorphisms and HLA-DRB1 alleles confer and increased risk of developing CV events in patients with RA.*

Introduction

Cardiovascular (CV) disease due to accelerated atherosclerosis constitutes the main cause of mortality in patients with rheumatoid arthritis (RA) (1). Both traditional and nontraditional risk factors contribute to this increased CV risk (2). In this regard, chronic inflammation and genetic susceptibility play a pivotal role in the development of accelerated atherosclerosis in RA (1-3). With respect to this, genes within the major histocompatibility complex (MHC), in particular some HLA-DRB1 alleles encoding a common sequence of amino acids corresponding to residues 67-74 within the HLA-DRβ1 chain, called shared epitope alleles, are associated with disease severity (4) including endothelial dysfunction (5) which is an early step in the atherogenesis process found in RA (6).

In line with the above, we found an increased incidence of CV events in RA patients carrying HLA-DRB1*04 shared epitope alleles. This was especially true for HLA-DRB1*0404 patients (3). In keeping with our observations, Mattey *et al.* has also described an increased risk of mortality due to ischemic heart disease in UK shared epitope positive RA patients, in particular in those carrying HLA-DRB1*0101/*0401 and 0404/*0404 genotypes (7). However, RA is a polygenic disease and the contribution of genes within the MHC to RA susceptibility only accounts for one-third to one-half of the total genetic contribution (8). Due to this, an important step forward in our understanding of the mechanisms leading to accelerated atherogenesis in RA may be to establish the potential contribution of other gene polymorphisms to the risk of CV events of patients with RA.

Nitric oxide (NO) is an important regulator of the Th1/Th2 balance in autoimmune diseases and peripheral blood mononuclear cells from RA patients have increased inducible NO synthase

(iNOS) expression and enhanced formation of NO that correlates with disease activity (9).

NO is produced constitutively by endothelial (eNOS or NOS3), or neuronal synthases (nNOS or NOS1), or in higher concentrations by iNOS (or NOS2) synthases after stimulation of a variety of pro-inflammatory cytokines (10). Several functional relevant polymorphisms in the *NOS2A* and *NOS3* genes have been identified, which have been associated with different vascular diseases including systemic vasculitides (11-15). In addition, we previously reported an association of the *NOS2A* $(CCTTT)_n$ repeat variations with RA susceptibility (16, 17).

On these bases, the human *NOS2A* and *NOS3* genes might be potential candidates for genetic association with CV events in RA.

Taking all these considerations into account, in the present study we sought to determine the potential contribution of the *NOS2A* and *NOS3* gene polymorphisms to CV events in a cohort of RA patients prospectively followed at the referral center for a well-defined area of Northwest Spain. Moreover, we also assessed whether potential interactions between *NOS* gene polymorphisms and *HLA-DRB1* alleles might increase the risk of developing CV events in RA.

Methods

Patients

One hundred and eighty-two consecutive patients who fulfilled the 1987 American College of Rheumatology classification criteria for RA seen at the Rheumatology outpatient clinic of Hospital Xeral-Calde, Lugo (Northwestern Spain) between March and September 1996 were included in this study (3). All subjects were prospectively followed and their clinical records were reviewed until patient's death or September 1, 2005. At the end of the study 39 (21%) patients had experienced CV events (3). Clinical information on this cohort of patients has recently been reported (3).

Detection of NOS2A and NOS3 polymorphisms

As previously reported, polymerase chain reaction (PCR)-based method combined with fluorescent technology was used for *NOS2A* $(CCTTT)_n$ genotyping (16, 17). The *NOS3 –786* and exon 7 variations were analyzed by PCR restriction fragment length polymorphism as previously described (17, 18).

Informed consent was obtained from all patients. The local institutional committee approved the study.

Statistical analysis

Strength of association between CV events in RA and alleles or genotypes of polymorphisms in the NOS2A and NOS3 genes was estimated using odds ratios (OR) and 95% confidence intervals (CI), via multiple logistic regression; estimates were adjusted by age at diagnosis of the disease (continuous), gender, age at the time of study (continuous), and traditional CV risk factors (presence/absence) as potential confounders. Statistical significance was defined as $p \leq 0.05$. Haplotype analysis of NOS3 gene polymorphisms was performed using the package SNPStats. Interactions between NOS gene polymorphisms and HLA-DRB1 alleles for the risk of developing CV events in RA were also estimated using OR and 95% CI, via multiple logistic regression. Calculations were performed with the statistical package Stata 10/SE.

Results

NOS2A promoter CCTTT repeat microsatellite polymorphism in RA

NOS2A $CCTTT_n$ allele and genotype frequencies were assessed in RA patients with and without CV events. The overall *NOS2A* $CCTTT_n$ allelic or genotypic distribution did not show statistical significant differences between both patient groups (data not shown). Likewise, when we stratified the *NOS2A* alleles in short (8-11) (fewer than 196 base pairs) and long (12-16) (196 base pairs or longer) repeats, no significant

Table I. Genotype frequencies of *NOS2A* and *NOS3* polymorphisms in RA patients with and without CV events.

Gene polymorphism	Genotype	Total no. (%)	Without CV events no. (%)	With CV events no. (%)
NOS3 (-786)	C/C	25 (15)	20 (15)	5 (14)
	C/T	79 (46)	67 (50)	12 (33)
	T/T	66 (39)	47 (35)	19 (53)
NOS3 (exon 7)	G/G	76 (43)	62 (44)	14 (39)
	G/T	79 (45)	60 (43)	19 (53)
	T/T	21 (12)	18 (13)	3 (8)
NOS2A $(CCTTT)_n$*	L/L	71 (39)	58 (41)	13 (33)
	L/S	77 (42)	58 (41)	19 (49)
	S/S	34 (19)	27 (19)	7 (18)

*S: Short (8-11 repeats); L: Long (12-16 repeats).

Table II. Association between *NOS2A* and *NOS3* gene polymorphisms and risk of CV events in RA patients (OR and 95% CI) adjusted by age at diagnosis of the disease, gender, age at the time of study, and traditional CV risk factors.

Gene polymorphism	Genotype	OR (95% CI)	*p*
NOS3 (-786)	C/C	0.71 (0.22-2.27)	
	C/T	0.50 (0.21-1.17)	
	T/T	1.00 (reference)	0.27
NOS3 (exon 7)	G/G	1.00 (reference)	0.61
	G/T	1.24 (0.55-2.80)	
	T/T	0.65 (0.16-2.60)	
NOS2A $(CCTTT)_n$*	L/L	1.00 (reference)	0.51
	L/S	1.52 (0.65-3.54)	
	S/S	0.91 (0.31-2.67)	

*S: Short (8-11 repeats); L: Long (12-16 repeats).

differences were observed between RA patients who experienced CV events or not. Table I shows *NOS2A* $CCTTT_n$ genotype frequencies in patients with or without CV events according to short or long alleles. Moreover, no increased risk of CV events was associated to this *NOS2A* gene polymorphism (Table II).

NOS3 gene polymorphisms in RA

NOS3 gene polymorphisms including a T/C polymorphism at position -786 in the promoter region and a polymorphism in exon 7 (298Glu/Asp) were also examined in RA patients who experienced CV events or not. However, no significant differences in allele and genotype frequencies between both groups of patients were observed. Table I shows genotype frequencies in this cohort of RA patients stratified by the presence of CV events. Also, no increased risk of CV events was associated to these *NOS3* gene polymorphisms (Table II). Likewise, no haplotype associations were observed when RA patients were stratified by the presence of CV events (data not shown).

Interactions between NOS2A promoter CCTTT repeat microsatellite or NOS3 gene polymorphisms and HLA-DRB1 alleles for the development of CV events

Since endothelial dysfunction and increased risk of CV events and CV mortality was more commonly observed in RA patients from Northwestern Spain carrying *HLA-DRB1*04* shared epitope alleles, in particular *HLA-DRB1*0404* (3, 5), RA patients with or without CV events were stratified according to *NOS* genotypes and *HLA-DRB1* status. Interestingly, as shown in Table III, some interactions between *NOS2A* promoter CCTTT repeat microsatellite or *NOS3* gene polymorphisms and *HLA-DRB1* alleles increased significantly the risk of developing CV events. In this regard, an increased frequency of CV events was observed in RA patients who were homozygous for the *NOS3 (-786) TT* genotype and carried the *HLA-DRB1*0404* allele (OR: 9.06 [95% CI: 1.29-63.37]; p=0.03). It was also the case for RA patients who were homozygous for the presence of long *NOS2A* alleles and carried the *HLA-DRB1*0404* allele (OR: 11.7 [95% CI: 1.53-88.4]; p=0.02) (Table III).

Discussion

CV events due to atherosclerosis are the leading cause of mortality in patients with RA (1, 3). Complex interactions between environmental and genetic determinants in both the host immune system and the vasculature may operate modifying the vascular risk in RA. In Northwestern Spain we previously reported an increased incidence of CV events in RA patients carrying *HLA-DRB1* 04* shared epitope alleles, in particular *HLA-DRB1*0404* (3). In this regard, the recognition of this genetic predisposition for CV disease (3, 5, 7) supports the search for additional genes that may be implicated in the increased incidence of CV complications observed in patients with this polygenic inflammatory autoimmune disease. However, a recent study on the macrophage inhibitory factor gene, previously associated with predisposition to RA and other rheumatic diseases, failed to find an association between CV disease and RA (19).

Endothelial dysfunction, an important surrogate marker of CV damage in early stages of the disease, is clinically present in RA (5, 6). Proinflammatory cytokines, in particular TNF-α, degrade eNOS mRNA and also block the activation of eNOS by interfering with the phosphorylation of protein kinase Akt (20). This fact leads to impairment in endothelial dependent vasodilatation.

In the present study, we determined the potential implication of *NOS2A* and *NOS3* gene polymorphisms in the increased risk of CV events associated to RA. With respect to this, although the results derived from the present study showed that *NOS2A* and *NOS3* gene polymorphisms do not infer a direct risk for CV events in patients with RA from Northwest Spain, interactions between the *NOS2A* promoter *CCTTT* repeat microsatellite or *NOS3 gene* polymorphisms and some *HLA-DRB1* alleles conferred and increased risk of developing CV events in these patients. In this regard, an increased risk of having CV events was found in RA patients who were homozygous for the *NOS3 (-786) TT* genotype or for the presence of long *NOS2A* alleles and carried the

Table III. Interactions between NOS2A promoter CCTTT repeat microsatellite or NOS3 gene polymorphisms and HLA-DRB1 for the risk of developing cardiovascular events (in all categories, patients not carrying the selected HLA-DRB1 alleles were used as reference, *i.e*. OR=1.0).

Gene			HLA-DRB1*04-SE#+		HLA-DRB1*0404+		HLA-DRB1*0401+		HLA- DRB1*0401 or *0404	
polymorphism	Genotype	n.	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
NOS3 (-786)	C/C	24	6.25 (0.13-301.7)	0.35	16.61 (0.11-2588)	0.28	1.29 (0.06-26.20)	0.87	0.70 (0.07-7.10)	0.76
	C/T	77	1.13 (0.29-4.36)	0.86	0.72 (0.06-9.51)	0.81	2.00 (0.47-8.46)	0.35	1.24 (0.38-4.05)	0.73
	T/T	65	3.50 (0.97-12.62)	0.06	9.06 (1.29-63.37)	0.03	1.82 (0.59-5.60)	0.29	2.80 (1.06-7.43)	0.04
NOS3 (exon 7)	G/G	75	3.06 (0.79-11.92)	0.11	6.89 (0.93-51.28)	0.06	4.82 (1.00-23.33)	0.05	2.94 (0.81-10.70)	0.10
	G/T	76	1.44 (0.47-4.39)	0.52	1.89 (0.29-12.28)	0.51	1.18 (0.36-3.86)	0.79	1.01 (0.27-2.77)	0.98
	T/T	21	4.76 (0.27-83.08)	0.29	4.42 (0.23-85.51)	0.33	1.77 (0.11-28.10)	0.69	4.98 (0.82-30.34)	0.08
NOS2A $(CCTTT)_n$ *	L/L	71	1.89 (0.52-6.87)	0.33	11.7 (1.53-88.4)	0.02	1.94 (0.54-7.04)	0.31	3.08 (1.04-9.15)	0.04
	L/S	77	3.60 (1.04-12.5)	0.04	1.45 (0.22-9.48)	0.70	2.30 (0.62-8.46)	0.21	1.17 (0.44-3.14)	0.76
	S/S	34	0.66 (0.12-3.81)	0.65	1.99 (0.13-29.5)	0.62	1.02 (0.14-7.27)	0.99	1.36 (0.23-7.90)	0.73

SE: Shared epitope.

*HLA-DRB1*0404* allele. This observation may suggest that the previously reported effect of *HLA-DRB1*0404* in terms of endothelial dysfunction and increased incidence of CV events may not be specifically due to this allele but the result of interactions with other genes located outside the MHC class II region.

In conclusion, our results suggest that gene-gene interactions may play an important role in the increased risk of developing CV events in patients with RA. The search for the potential implication of other gene polymorphisms in the risk of CV events in RA is required to better establish the genetic basis of the mechanisms leading to accelerated atherosclerosis in this complex autoimmune disease.

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