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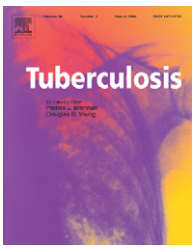

A polymorphism in the inducible nitric oxide synthase gene is associated with tuberculosis

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Summary
iNOS or NOS2 is a molecule that plays a key role in the immunological control of a broad spectrum of infectious agents. Investigation is hampered by difficulty in estimating *in vivo* production of nitric oxide (NO), but genetic studies provide a potential means of examining the relation between NO production and disease outcome. To better characterize the host genetic factors determining the susceptibility to TB, we evaluated the influence of two polymorphisms in the *NOS2A* gene on the risk of developing pulmonary TB in a Northwestern Colombian population, which is a moderately-high endemic area. One hundred and fourteen patients with TB and negative for human immunodeficiency virus, plus 304 healthy controls were examined for *NOS2A* CCTTT and TAAA polymorphisms. A total of 160 healthy controls mentioned before, underwent tuberculin skin test (TST). Analysis disclosed significant differences between patients and controls with *NOS2A* CCTTT polymorphism ($P = 0.0001$, $Pc = 0.001$, OR = 0.4, and 95%CI = 0.3–0.7) independent of TST status. When the *NOS2A* alleles were stratified into short (8–11) and long (12–16) repeats, significant differences with short repeats were observed between TB patients and all controls ($P = 0.005$, OR = 0.63, 95%CI = 0.46–0.86). No individual association with *NOS2A* TAAA was detected. These results indicate that a polymorphism in the *NOS2A* gene

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influences the susceptibility to TB and suggest a role for *NOS2A* in the pathogenesis of mycobacterial infection.

Introduction

There has been an explosion of information about nitric oxide (NO) which appears to be involved in an extraordinary range of functions including vascular regulation, neurotransmission, cytotoxicity, and host defense.[1] NO is formed when the guanido group of the essential amino acid L-arginine is split forming NO and L-citrulline. The reaction is catalyzed by a class of enzymes called NO synthases (NOS). Three species of NOS have been characterized and their NOS genes have been identified.[2] NO is produced constitutively by endothelial (eNOS or NOS3) or neuronal (nNOS or NOS1) synthases, or in higher concentrations by inducible (iNOS or NOS2) synthases.[3] NOS2 is induced by several cytokines including IFN-γ, IL-1β, and TNF-α as well as by endotoxins in inflammation.[3]Induction of NOS2 results in the production of a larger amount of NO and may contribute to host defense against the infection by bacteria and parasites.[2]

In the murine model of tuberculosis (TB), NO plays an essential role in the killing of *Mycobacterium tuberculosis* by mononuclear phagocytes.[4] Intratracheal administration of virulent *M. tuberculosis* to rats stimulated NOS2 and NO production in alveolar macrophages.[5] The best examples of the protective role of NO in murine TB is illustrated by the genetically disrupted *NOS2A* mouse strain (iNOS $-/-$), where infection with *M. tuberculosis* was associated with a significantly higher risk of dissemination and mortality compared with wild type mice.[4]

In contrast to murine models of TB, there is greater controversy about the role of NO in killing or limiting the growth of *M. tuberculosis* in humans. It has been proposed that NO produced by TB-infected human macrophages and by epithelial cells is also antimycobacterial against *M. tuberculosis*.[6,7] Nevertheless, several reports indicate that alveolar macrophages from TB patients produce increased amounts of NO compared to healthy control subjects.[8–10] Wang et al.[10] demonstrated that the increased NO exhalation in patients with TB was due to the up-regulation of *NOS2A* in alveolar macrophages. In contrast to the NO function inferred from mice (as a mycobactericidal molecule), it may also participate in the formation of protective granulomas.[11]

Although NOS2 expression is not usually detected in human macrophages from cell lines or macrophages derived *in vitro* from blood monocytes of healthy humans, alveolar and tissue macrophages from lungs of TB patients show high levels of functional NOS2.[8] In addition, infected lung tissue from TB patients shows high levels of nitrotyrosine, a product of NOS activity, as well as increased NOS2 activity.[12]

NOS2 is encoded by a polymorphic gene known as *NOS2A* at chromosome 17q11.2–q12. Several single nucleotide polymorphisms (SNPs) and microsatellites have been described. In spite of some conflicting results, a highly polymorphic pentanucleotide (CCTTT)n microsatellite located in the *NOS2A* gene promoter region has been indicated to be functionally important in the regulation of the *NOS2A* transcription.[13–17] Also, associations of the (CCTTT)n microsatellite have been reported in autoimmune and infectious diseases.[18–20] In addition, a polymorphism in the proximal promoter involving the insertion or deletion of a unit of a TAAA repeat[21–23] has been shown to be associated with an increased risk of inflammatory abnormalities.

The *NOS2A* gene is located in a region that has been linked to susceptibility to TB.[24,25] Given the biological and genetic plausibility of *NOS2A* in the immune response to TB, the aim of this study was to examine the influence of *NOS2A* polymorphisms on the risk of developing TB in a Northwestern Colombian population, which is a moderately high endemic area for TB.[26]

Materials and methods

Study population

One hundred and fourteen patients with pulmonary TB were enrolled in the study at the time their disease was treated. Their mean age was 39.5 ± 16.8 (Table 1). TB was diagnosed by the presence of acid-fast bacilli in sputum or isolation of *M. tuberculosis* in culture. In all cases, TB patients were negative for HIV 1/2 infection (by Axsym assays, Abbott Laboratories, Chicago, USA). Patients had been enrolled at the Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia, as part of a project on the immunogenetics of TB.[27]

As a control population, 304 individuals over the age of 18, from both genders, without inflammatory, autoimmune diseases or a history of chronic infectious diseases, including TB and HIV infection were involved (Table 1). A representative number of controls were stratified according to tuberculin skin test (TST). All controls were matched to patients by gender, ethnicity and socioeconomic status and were unrelated to the patients.

Our population belongs to a genetic isolate—the Paisa community in Colombia, South America. Extensive descriptions of the Paisas have been published elsewhere.[28,29] Briefly, the Paisa community, which contains over 4 million inhabitants, is located between the Central and Western branches of the Andean mountain range and was geographically isolated from the 16th until the second half of the 20th century. Historically, the Paisas descended largely from Spaniards and Sephardic Jews.[28,29]

Tuberculin skin test

A total of 160 healthy controls underwent testing to assess the delayed type hypersensitivity (DTH) skin test response to 0.1 ml (5TU) of the purified protein derivate (PPD) antigen (Tuberculin PPD powder master lot #154616, from Public Health Service, National Center for Disease Control, USA)

Table 1 Clinical detail of individuals in the case-control study.

Characteristic	TB patients ($n = 114$)	All controls ($n = 304$)	TST+ controls ($n = 78$)	TST− controls ($n = 82$)
Age, mean (SD), years	39.5 (16.8)	44.5 (15.1)	46.6 (13.8)	42.7 (15.9)
Gender				
Male	52 (45.6)	120 (39.5)	20 (44.4)	25 (55.5)
Female	62 (54.4)	184 (60.5)	49 (49)	51 (51)
Presence of extrapulmonary manifestations[a]	27 (23.7)	NA	NA	NA

Abbreviations: TST: tuberculin skin test; NA: no data; HIV: human immunodeficiency virus; TB: tuberculosis.
[a]All patients had pulmonary tuberculosis but some of them developed extra-pulmonary manifestation such as pleura, larynx, bone and kidney involvements.

injected intradermally on the forearm, following the Mantoux method.[30] The skin test response was measured as the diameter of induration 48–72 h after injection. These subjects were classified into those who were naturally infected with *M. tuberculosis* (i.e. diameter of the induration was $\geqslant 10$ mm) and those who were uninfected at the time of DTH ascertainment (diameter of the induration was <10 mm); 78 (~48.8%) subjects were considered to be naturally infected while 82 (~51.2%) were TST negative and thus considered non-infected. The mean age of these individuals was 46.6 ± 13.8 years for TST+ healthy controls and 42.7 ± 15.9 years for TST-healthy controls (Table 1). All of them were negative for TB disease (i.e. absence of respiratory symptoms and normal chest X-ray).

Genotyping

Genomic DNA was isolated from 10 ml of EDTA-anticoagulated blood samples using the standard salting-out technique. A polymerase chain reaction (PCR)-based method combined with fluorescent technology was used for CCTTT and TAAA genotyping as previously described.[31] The forward and reverse primers used were 5′ TGC CAC TCC GCT CCA G 3′ and 5′ GGC CTC TGA GAT GTT GGT CTT 3′ for $TAAA_n$ and 5′ ACC CCT GGA AGC CTA CAA CTG CAT 3′ and 5′ GCC ACT GCA CCC TAG CCT GTC TCA 3′ for $CCTTT_n$. The forward primers were 5′ labeled with the fluorescent dye 6–FAM. After capillary electrophoresis on an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems), alleles of the CCTTT and TAAA repeat elements were identified using the Genotyper 3.7 software (Applied Biosystems). For quality control of the genotyping, 10% of the samples were sequenced to confirm the length of each allele. The primers used for sequencing were the same as those used for the capillary electrophoresis.

Statistical analysis

Allele and genotype frequencies were obtained by direct counting. Hardy–Weinberg equilibrium and statistical analysis to compare allelic and genotypic distributions were performed using two-sided χ^2 and Fisher's exact test, as appropriate. Odds ratios (OR) with 95% confidence intervals (95%CI) were calculated according to Woolf's method and a P-value <0.05 was considered to be statistically significant. P-values were corrected by the number of comparisons using Bonferroni correction. Data were managed using the GraphPad InStat V3.00 for Windows (GraphPad Software, San Diego CA, USA). Population heterogeneity among the case and control sets was examined using Wright's F-statistics according to the non-biased method of Weir and Hill.[32]

This research was conducted in compliance with resolution 008430 of 1993 of the Colombian Ministry of Health, and was classified as research with minimal risk. The local ethics committee approved the study.

Results

Genetic structure analyzes

Hardy–Weinberg equilibrium was noticed in all samples. A high degree of linkage disequilibrium was observed between *NOS2A* CCTTT pentanucleotide repeats and *NOS2A* TAAA insertion/deletion (data not shown). Population stratification was not registered in our sample since the Fst subdivision coefficient was not significantly different from 0 (data not shown). Thus, patients and controls were from a similar genetic background. The allele frequencies within the control group showed a unimodal distribution, similar to that found in the Caucasian population, but different from the bimodal curve reported in African-Caribbean and Gambian populations.[33,34]

NOS2A CCTTT repeat microsatellite polymorphism

Ten *NOS2A* CCTTT alleles were observed in our population. Determination of allele frequencies showed that the most frequent alleles in the CCTTT repeat microsatellite were the 10-repeat allele (25%), 11-repeat allele (24.8%) and 9-repeat allele (16.6%). The overall $CCTTT_n$ distribution showed statistically significant differences between TB and all controls ($P = 0.017$) (Table 2). This association was also observed when TB versus TST+ controls were compared ($P = 0.016$) (Table 2). When individual CCTTT alleles were analyzed, a significant decrease in frequency of the 10-repeat single allele was observed in the cases (12.7%) compared with controls (25%) ($P = 0.0001$, $Pc = 0.001$,

Table 2 Allele frequencies of NOS2A (CCTTT)n gene polymorphism in TB patients and controls.

Repeat no.	Size (base pair)	TB $2n = 228$ (%)	All controls $2n = 608$ (%)	TST+ controls $2n = 156$ (%)	TST− controls $2n = 164$ (%)
7	171	9 (3.9)	9 (1.5)	2 (1.3)	3 (1.8)
8	176	8 (3.5)	20 (3.3)	3 (1.9)	4 (2.4)
9	181	33 (14.5)	101 (16.6)	31 (19.9)	28 (17.1)
10	186	29 (12.7)	152 (25)	39 (25)	41 (25)
11	191	60 (26.3)	151 (24.8)	37 (23.7)	39 (23.8)
12	196	36 (15.8)	67 (11)	16 (10.3)	19 (11.6)
13	201	24 (10.5)	45 (7.4)	14 (9)	11 (6.7)
14	206	25 (11)	45 (7.4)	9 (5.8)	13 (7.9)
15	211	3 (1.3)	17 (2.5)	5 (3.2)	5 (3)
16	216	1 (0.4)	1 (0.2)	0 (0)	1 (0.6)

Comparison between TB patients and all controls, χ^2: 26.46, $P_{(2\times10)} = 0.017$.
Comparison between TB patients and TST+ controls, χ^2: 20.28, $P_{(2\times10)} = 0.016$.
Comparison of the 10-repeat single allele (186 base pairs) versus all other alleles between TB patients and all controls, $P = 0.0001$, $Pc = 0.001$, OR = 0.4, and 95%CI = 0.28–0.67.

Table 3 Stratification according to short and long forms of NOS2A CCTTT marker between patients and healthy controls.

Alleles	TB $2n = 228$ (%)	All controls $2n = 608$ (%)	TST+ controls $2n = 156$ (%)	TST− controls $2n = 164$ (%)
Short (7–11 repeats)	139 (61)	433 (71.2)	112 (71.8)	115 (70.1)
Long (12–16 repeats)	89 (39)	175 (28.8)	44 (28.2)	49 (29.9)

The frequency of short alleles (7–11 repeats) was significantly decreased in TB patients compared to all controls, $P = 0.005$, OR = 0.63, 95%CI = 0.46–0.86.

OR = 0.4, and 95%CI = 0.28–0.67) independently of TST status. Interestingly, when the *NOS2A* alleles were stratified into short (8–11) and long (12–16) repeats, significant differences with short repeats were observed between TB patients and all controls (Table 3).

NOS2A TAAA repeat polymorphism

The presence of insertion/deletion of TAAA was observed in our population. The most frequent allele of TAAA polymorphism was 220 base pairs allele. Although the frequency of the *NOS2A* 224 allele was higher in TB patients (11%) compared to all controls (7.1%), no statistically significant allele or genotype differences between TB patients and all controls for this *NOS2A* polymorphism were found (Table 4). Nor were significant differences between TB patients and TST stratified controls observed.

Clinical features of TB patients were analyzed with the different alleles, genotypes or haplotypes. In this regard, no statistically significant differences were observed in the distribution of *NOS2A* polymorphisms. Similarly, differences between these polymorphisms were not affected by gender.

Discussion

NOS2A gene is located in 17q11.2, a region linked to TB and also known to carry susceptibility genes for intra-macrophage pathogens such as *M. tuberculosis* and *Mycobacterium leprae*.[24,35] Our results showed that the *NOS2A* CCTTT 10-repeat single allele (186 base pairs) confers resistance to TB in Northwestern Colombians. Interestingly, this polymorphism has been investigated previously in a Brazilian population with a significant global association between the variant and susceptibility to TB being reported.[24]

Results from case-control studies may be influenced by population stratification and low power to detect true associations. In this regard, Fst parameter was calculated to examine whether or not our study showed true statistical differences or differences that were due to stratification. The results were not statistically different from 0, which indicated that the cases and controls had a similar genetic background. Our sample size was large enough to detect an association for *NOS2A* CCTTT microsatellite at an OR between 0.1 and 0.5 since it had 99% power at the 5% significance level.

The unimodal distribution of CCTTT microsatellites confirms the highly significant differences reported in the *NOS2A* allele frequencies between ethnically diverse populations with regards to the *NOS2A* CCTTT pentanucleotide microsatellite, suggesting, although not formally demonstrating, that variation in the number of CCTTT repeats in the *NOS2A* gene may have some significance in the predisposition of the human population to infectious diseases. Thus, a founder effect together with selective pressure account for the observed unimodal distribution of CCTTT alleles.[33] A unimodal distribution pattern was observed as previously reported in Caucasian populations.[33,34] In spite of the similarity in pattern distribution, we did not find the same most common allele. For

Table 4 Allele and genotype frequencies of NOS2A TAAA polymorphism in TB patients and healthy controls.

	TB patients $n = 114$ (%)	All controls $n = 296$ (%)	TST+ controls $n = 80$ (%)	TST− controls $n = 78$ (%)
Genotype				
220/220	90 (78.9)	254 (85.8)	66 (82.5)	67 (85.9)
220/224	23 (20.2)	42 (14.2)	14 (17.5)	11 (14.1)
224/224	1 (0.9)	0 (0)	0 (0)	0 (0)
Allele	$2n = 228$	$2n = 592$	$2n = 160$	$2n = 156$
220	203 (89)	550 (92.9)	146 (91.2)	145 (92.9)
224	25 (11)	42 (7.1)	14 (8.8)	11 (7.1)

No statistically significant differences were found in any comparisons.

Caucasians, the most common allele was 12 repeats instead of 10 and 11 repeats for Northwestern Colombians.

The microsatellites are potentially unstable but highly informative markers. Some microsatellites may affect the transcription or level of protein expression. NOS2A CCTTT microsatellite is located in an S1 hypersensitivity region ~2.5 kb upstream of the human *NOS2A* gene transcription start site. This polymorphism may form an unusual structure of triplex DNA and affect the transcription.[15,36] In addition, *in vitro* data have defined a functional effect for the *NOS2A* CCTTT microsatellite.[13]

Burgner et al.[37] described the relationship between SNPs and microsatellite markers at the *NOS2A* locus in two populations. Although the *NOS2A* SNPs analyzed are not in LD with the CCTTT microsatellite, a possible LD in our population between the *NOS2A* CCTTT microsatellite repeats and another polymorphic site in the vicinity can not be discarded.

Other polymorphisms in the *NOS2A* gene promoter have been described. One of these, the G-954C variant was originally reported in a highly endemic African malaria areas[9] suggesting that this mutation originated as a consequence of the selective pressure of *Plasmodium*. Of note, the G allele has been shown to be absent from Caucasian populations[38,39] and has previously been reported to be absent from the Peruvian population.[40] In Mexicans, this SNP was not associated with TB in whom the frequency of G allele was ~5%.[41] The G-954C variant was not considered in our study due to the low frequency of the minor allele reported in other Latin American populations and we believe that this SNP is not for examining the influence of the *NOS2A* gene in TB in our population. The microsatellite CCTTT was selected as a highly polymorphic marker (14 alleles have been described with heterozygosity of 0.80) with the potential to affect NOS transcription.[13–16]

Genetic studies might provide a potential means of examining the relationship between NO production and disease outcome. *NOS2A* CCTTT individual alleles have been associated with several inflammatory diseases such as diabetic retinopathy,[13] certain forms of dementia,[42] atopia[43] and systemic lupus erythematosus in African-Americans.[44]

On the other hand, carriers of long repeats, who are prone to produce higher amounts of NO, are at greater risk than non-carriers.[13] According to this, the results are in agreement with functional studies in which the macrophage and other cells inside of the granuloma often express more NOS2 than controls.[8,45,46] The role of NO in host defense against human TB is controversial. Although experimental evidence indicates that NO may play an important role in controlling TB[4,47] there is greater controversy over the role of NO in killing or limiting the growth of *M. tuberculosis* in humans. An increased NOS2 protein expression in alveolar macrophages from TB patients has been demonstrated. Moreover, it showed that *NOS2A* was catalytically active, providing proof that there was high-output NO production in TB-infected macrophages.[8] The question that arises is what the role of NO in physiopathology of TB is. Kuo et al.[9] also showed that alveolar macrophages from TB patients produced increased amounts of NO compared to healthy control subjects. Furthermore, NO played an autoregulatory role in amplifying the synthesis of TNF-α and IL-1β.[9] Wang et al.[10] demonstrated that the increased NO exhalation in patients with TB was due to up-regulation of NOS2 in alveolar macrophages. In addition, the amount of exhaled NO correlated with the capacity of the alveolar macrophages *in vitro* to produce NO. NO is not only mycobactericidal but may also participate in the formation of protective tissue granulomas.[11]

In summary, we showed that *NOS2A* gene influences the susceptibility/resistance for developing TB in a Northwestern Colombian population.

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