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# ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE INTRON- 4 27BP REPEAT POLYMORPHISM IS NOT ASSOCIATED WITH RHEUMATOID ARTHRITIS AMONG ASIAN INDIANS

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## ABSTRACT

The aim of this study was to investigate the association of intron- 4 27bp repeat polymorphism in the endothelial nitric oxide synthase (eNOS) gene with rheumatoid arthritis (RA) in Asian Indians. A case control study in which 100 patients diagnosed with RA and 100 healthy controls were enrolled. DNA was extracted from peripheral blood and eNOS polymorphism was detected by PCR. All study participants were screened for nitric oxide (NO).Allelic and genotypic distribution did not differ significantly between RA patients and healthy control subjects. Reduced NO levels was detected in patients as compared to controls. Patients with bb genotype and ab genotypes were found to have significantly lower level of plasma NO as compared to controls with the same types of genotypes. eNOS gene intron- 4 27bp repeat polymorphism is not associated with the susceptibility to RA in Asian Indians.

**KEYWORDS: Rheumatoid Arthritis, eNOS gene polymorphism**

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# INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common systemic inflammatory diseases. However, the exact mechanism of the disease is not clear [1]. Some studies suggested that nitric oxide (NO) plays an important role in the pathogenesis of RA. The level of NO is elevated in RA patients [2], and another study suggested that NO can regulate the balance of Th1/Th2 in autoimmune diseases, and it was a key mediator of apoptosis within RA joints [3]. NO synthesis is tightly regulated by three forms of nitric oxide synthase (NOS) including neuronal synthases (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Several functionally relevant polymorphisms in the iNOS and eNOS genes have been identified, which have been associated with many diseases, such as vascular disease, autoimmune disease, and infectious diseases [4]. The eNOS gene is located on chromosome 7q35-q36, and is a potential candidate gene in the regulation of the immune reaction as it can influence the production of NO and the risk of rheumatoid arthritis. One of the common polymorphisms in the eNOS gene is 27bp repeat polymorphism in intron 4 of the gene. Many studies have shown the association between eNOS gene polymorphism and some autoimmune diseases. However, the results of those studies are conflicting [5]. To date, no reports are available for the association of eNOS gene intron-4 27bp repeat polymorphism with RA in the Asian Indian population. Hence, the aim of our study was to examine the possible association between the eNOS gene intron-4 27bp repeat polymorphism and RA among Asian Indian population.

# MATERIALS AND METHODS

### *Subjects*

One hundred rheumatoid arthritis patients were recruited from the Department of Rheumatology (Medicine) of the All India Institute of Medical Sciences (AIIMS), New Delhi. All the patients met the American College of Rheumatology 1987 revised criteria[6], were 20 to 60 years old (mean age (39.05±11.45) years). All the patients were on treatment for RA before recruitment. One hundred age- and ethnicity-matched normal volunteers comprising patient's relatives, students of AIIMS and voluntary blood donors of Blood Bank, AIIMS were studied as controls. All the controls were 20 to 60 years old (mean age (29.5±7.42) years). Each participant donated peripheral blood for DNA analysis and plasma isolation. Plasma samples were stored at −20◦C in aliquots until use. All study participants provided written informed consent and the study was approved by the local ethics committee.

### *DNA extraction*

Genomic DNA was isolated from peripheral blood leucocytes by the Miller extraction method [7].

### *Analysis of the 27bp repeats polymorphism in intron 4 of the e NOS gene*

Detection of 27bp repeat polymorphism in intron 4 of the e NOS gene was performed in all the subjects by PCR genotyping. Primer pairs used were as follows: sense-5' AGG CCC TAT GGT AGT GCC TTT 3' and antisense- 5' TCT CTT AGT GCT GTG GTC AC 3'. Samples were amplified for 36 cycles, consisting of denaturation at $95^0$C for 45 seconds, annealing at $56^0$C for 1 minute and extension at $72^0$C for 1 minute with a final extension of 7 minutes. Amplified products were run on 2.5% agarose gels and visualized by ethidium bromide staining. 420bp size product denotes 'b' allele (five repeats) and 393 bp size product denotes 'a' allele (four repeats).

### *Estimation of nitric oxide (NO)*

Plasma NO levels was evaluated using sodium nitrite ($NO_2$) as standard by acidic Griess reaction method [8].

### *Statistical Methods*

Statistical analysis were performed according to SPSS(Statistical Package for Social Sciences) for windows(version 9.0.0, SPSS Inc., Chicago) and TFPGA (Tools for population genetic analysis) version 1.3

developed by Mark Miller from the department of biological science, North Arizona University. Frequency of genotypes (bb,ab, aa) and alleles (b,a) of 27bp repeat polymorphism in intron 4 of eNOS gene were assessed using Fisher's exact test and chi-square test wherever applicable. Mann Whitney test and Kruskal Wallis test were used wherever applicable. Unpaired and two tailed t tests were used to analyze laboratory data. P value ≤0.05 was considered statistically significant.

# RESULTS

***Association of eNOS gene intron-4 27bp repeat Polymorphism and Rheumatoid arthritis***

On comparative evaluation of the frequency of the intron-4 27bp repeat Polymorphism genotype and alleles between the patients and healthy subjects (table 1), it was found that b allele and bb genotype were more prevalent in RA patients (0.84 & 71%) as compared to healthy subjects (0.75 & 58%). The OR of bb genotype was 1.77 (95% CI= 0.95-3.33, p=0.055) to develop Rheumatoid arthritis. The OR of ab genotype was 0.68 (95% CI= 0.35-1.31, p=0.22) to develop Rheumatoid arthritis. The OR of aa genotype was 0.36 (95% CI= 0.07-1.53, p=0.12) to develop Rheumatoid arthritis.

**Table 1**
***Distribution of eNOS VNTR intron 4 a/b Genotypes and Frequency of Alleles in Controls and Rheumatoid arthritis Patients***

| | Patients (N=100) | Controls (N=100) | P value | O.R. | C.I. |
|---|---|---|---|---|---|
| Genotypes | | | | | |
| bb | 71 | 58 | 0.055 | 1.77 | 0.95 - 3.33 |
| ab | 26 | 34 | 0.22 | 0.68 | 0.35 – 1.31 |
| aa | 3 | 8 | 0.12 | 0.36 | 0.07 – 1.53 |
| ab+aa | 29 | 41 | 0.08 | 0.59 | 0.31 – 1.10 |
| Alleles | | | | | |
| b | 0.84 | 0.75 | | | |
| a | 0.16 | 0.25 | | | |

***Levels of NO in controls and patients***

Table 2 shows the values of Nitric oxide (NO) for controls and patients. The value for NO in controls and patients ranged from1.45 to 65.4 and 1.38 to 57.27 µmol/l with median of 14.78 and 9.56 respectively. Mean values for NO in controls was 18.54 µmol/l and that in patients were significantly low (p<0.001).

**Table 2**
***Plasma levels of NO in controls and RA patients***

| | *CONTROL* | | | *PATIENTS* | | | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Mean± SD** | **median** | **Range** | **Mean ± SD** | **median** | **Range** | **P value** |
| NO | 18.54± 13.5 | 14.78 | 1.45-65.04 | 11.83± 10.25 | 9.56 | 1.38-57.27 | 0.001 |

***Relationship of eNOS gene intron-4 27bp repeat polymorphism and plasma NO in controls and patients***

Plasma levels of NO in individual genotypes in controls and patients are shown in Table 3. As shown in Table 3, patients with bb and ab genotypes were found to have significantly low levels of plasma NO as compared to controls with the same type of genotypes (p < 0.05). Whereas there was no significant difference in plasma NO levels of controls and patients with aa genotype (p > 0.05). There was no significant difference in plasma NO levels among patients with bb, ab and aa genotypes (p> 0.05). Likewise, there was no significant difference in plasma NO levels among controls with bb, ab and aa genotypes (p>0.05)

**Table 3**
***Plasma NO levels in relation to eNOS intron 4 a/b polymorphism***

| PATIENTS | | | CONTROLS | | | |
|---|---|---|---|---|---|---|
| **Genotype** | **Median** | **Mean± SD** | **Genotype** | **Median** | **Mean± SD** | **P value** |
| bb (71) | 9.36 | 10.8 ± 8 | bb (58) | 14.78 | 17.33 ± 11.73 | 0.001 |
| ab (26) | 9.11 | 14.45 ± 14.73 | ab (34) | 16.88 | 20.14 ± 15.55 | 0.046 |
| aa (3) | 14.61 | 13.29 ± 10.96 | aa (8) | 13.46 | 20.5 ± 17.32 | 0.84 |

# DISCUSSION

In this study, we investigated whether the eNOS gene intron-4 27bp repeat polymorphism could be implicated in susceptibility to RA. It was found that there was no significant difference in the frequencies of bb, ab and aa genotypes of the eNOS gene intron 4 among controls and RA patients. However, the distribution pattern obeyed Hardy Weinberg equilibrium thereby suggesting that the sample size was appropriate for the study on eNOS gene intron-4 27bp repeat polymorphism. This finding is in contrast to a previous finding referred to a Cretan cohort of RA patients implicating the polymorphism in susceptibility to RA[9]. This may be due to the differences in the genetic background between the populations studied. We demonstrated that the plasma NO levels were significantly lower in RA patients as compared to healthy controls. This finding is in contrast to a previous study where the level of NO was found to be increased in rheumatoid arthritis patients [2]. This may be due to the fact that in our study all the patients were on treatment for RA before recruitment. Therefore one of the strategies might be recruitment of only treatment naive RA patients in future studies. In our study, plasma NO levels were correlated with eNOS gene intron-4 27bp repeat polymorphism. It was found that RA patients with bb and ab genotypes were found to have significantly lower levels of plasma NO as compared to controls with the same type of genotypes. However, we have demonstrated that there was no significant difference in the frequencies of bb, ab and aa genotypes of the eNOS gene intron 4 among controls and RA patients. At present, we cannot provide a reasonable explanation for this apparently contrasting finding. In conclusion, we observed lack of association between eNOS gene intron-4 27bp repeat polymorphism and risk of RA in Asian Indians. It is apparent that this is the first case-control study that has evaluated the risk associated with eNOS gene intron-4 27bp repeat polymorphism with RA in an Asian Indian population. Larger studies in other ethnic populations are warranted to determine the role of this polymorphism in the etiology of RA.

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***Conflict of interest***
Conflict of interest declared none**.**

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