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Research paper

Association of *Nitric Oxide Synthase2* gene polymorphisms with leprosy reactions in northern Indian population

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ABSTRACT

The pathogen *Mycobacterium leprae* causes leprosy that affects mainly skin and nerves. Polymorphisms of certain genes are substantiated to be associated with the susceptibility/resistance to leprosy. The present investigation addressed the association of *Nitric Oxide Synthase2* gene polymorphisms and leprosy in a population from northern part of India. A total of 323 leprosy cases and 288 healthy controls were genotyped for four *NOS2* promoter variants (rs1800482, rs2779249, rs8078340 and rs2301369) using FRET technology in Real Time PCR. None of these SNPs in promoter sites was associated with susceptibility/resistance to leprosy. *NOS2* rs1800482 was found to be monomorphic with GG genotype. However, *NOS2-1026T* allele was observed to be in higher frequency with leprosy cases (BL and LL) who were not suffering from any reactional episodes compared to cases with ENL reaction {OR = 0.30, 95% CI (0.10–0.86), p = 0.024}. *NOS2-1026GT* genotype was more prevalent in cases without reaction (BT, BB and BL) compared to RR reactional patients {OR = 0.38, 95% CI (0.17–0.86), p = 0.02}. Although haplotype analysis revealed that no haplotype was associated with leprosy susceptibility/resistance with statistical significance, GTG haplotype was noted to be more frequent in healthy controls. These SNPs are observed to be in linkage disequilibrium. Although, these SNPs are not likely to influence leprosy vulnerability, *-1026G > T* SNP was indicated to have noteworthy role in leprosy reactions.

1. Introduction

Leprosy is caused by *Mycobacterium leprae* (*M. leprae*). The disease affects the population in their productive age and thus exerts a major economic and social problem on the society (Britton and Lockwood, 2004). Even though effective eradication programme is continuing; leprosy is still prevalent in several developing countries such as Africa, Asia and South America and in Brazil. A total of 174,608 registered and 210,758 new cases were detected at the end of 2015 (WHO Report, 2016).

Leprosy comprises of a broad range of clinical manifestations from tuberculoid (TT) pole to lepromatous leprosy (LL) pole, borderline forms {borderline tuberculoid (BT), borderline (BB), and borderline lepromatous (BL)} in between, based on the host immune responses against *M. leprae* (Ridley and Jopling, 1966). On tuberculoid pole, paucibacillary patients show a strong delayed type of hypersensitivity (DTH) response while patients of lepromatous pole with large number of bacteria exhibit anergy to *M leprae* antigens in skin test and lymphocyte transformation test (Myrvang et al., 1973). Leprosy patients often experience clinical complications during the course of treatment known as reactions. Mainly two types of reactions occur in leprosy, Type 1 or Reversal Reaction (RR) and Type 2 or Erythema nodosum leprosum (ENL) causing medical emergency. During this phase due to severe nerve injury which may develop quickly with loss of sensation, deformity and paralysis (Becx-Bleumink and Berhe, 1992). Borderline group of patients (i.e., BL, BB, and BT) are often found to go through Type 1 reaction while multibacillary patients (BL and LL), experience

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Type 2 reaction (Kumar et al., 2004). Many recent studies support that change in host response to leprosy infections has effective genetic basis. In different geographical populations of the world, association of genetic variants with resistance/susceptibility to leprosy was exhibited (Ali et al., 2012; Boldt et al., 2013; de Messias-Reason et al., 2014; de Messias-Reason et al., 2009; de Messias-Reason et al., 2007; Jamieson et al., 2004; Marcinek et al., 2013; Mira et al., 2003; Shinde et al., 2013; Zhang et al., 2011, 2009).

Host immune responses are mediated by many components. Nitric Oxide (NO) acts as an important mediator for killing of pathogens during innate immune responses (Bogdan, 2001). NO is produced in activated macrophages by the enzyme, *Nitric Oxide Synthase* (*NOS2*) (Nathan, 1992). The chromosome 17q11.2-12 encodes the protein *NOS2* of 131 kDa and has a genomic size of 48 kb (Xu et al., 1996). The inflammatory cytokines produced during infection and injuries regulate *NOS2* expression at transcriptional level (Nathan, 1997). NOS2 expression was reported in lesions of borderline leprosy patients (Khanolkar-Young et al., 2001; Schon et al., 2001). Increased urinary NO metabolites were also detected in leprosy reactional patients (Mohanty et al., 2007). Promoter polymorphisms in *NOS2* gene have been attributed to influence the susceptibility in Tuberculosis in Brazilian population (Jamieson et al., 2004), north western Columbian population (Gomez et al., 2007), African Americans (Velez et al., 2009). *NOS2G-954C* was reported to alter the *NOS2* activity in polymorphic individuals (Kun et al., 2001).

As during infection and inflammation, NO production was linked with important immunological consequences and leprosy is associated with inflammatory responses, we hypothesize that diverse clinical phenotypes in patients with leprosy and leprosy reactions may be associated with the common genetic variations occur in the *NOS2* promoter gene.

2. Materials and methods

2.1. Ethics statement

The procedures of this study were approved by Institutional human ethics committee. This committee was formed as per the guidelines set by Indian Council of Medical Research (ICMR, 2006).

2.2. Study population

Leprosy cases who were attending the outpatient department (OPD) of National JALMA Institute for Leprosy and Other Mycobacterial Diseases from Monday to Friday were first informed about the study. Three hundred twenty three patients, those who full filled the inclusion criteria were included. The written informed consent was obtained from all participants. In case of children, written informed consent was obtained from a parent or guardian. Patient information sheets detailing their age, sex, clinical information, and socio economic information were filled in after interacting with the participants. The clinical features of the cases and healthy controls were described in Table 1.

Table 1
Demographic characteristic of leprosy cases and controls.

Subject	Total number	Male	Female	Average age ± SD
Controls[a,b]	288	242	46	37.42 ± 13.04
Cases	323	228	95	35.70 ± 14.01
TT	7			
BT	69			
BB	101			
BL	57			
LL	74			
Neuritic	15			
ENL	45			
BL	8			
LL	37			
RR	65			
BT	7			
BB	38			
BL	20			

[a] Mean age of total leprosy cases and healthy controls was compared by student's *t*-test, $p = 0.119$.
[b] Male: female ratio was compared between total leprosy cases and controls by chi-square test, $p = 0.000082$.

2.2.1. Leprosy cases

Leprosy cases, from 7 to 80 years of age were diagnosed clinically on the basis of skin patches, nerve thickening, sensation testing and bacteriologically by slit skin smear test. Patients were divided into a five-group spectrum according to the Indian Association of Leprologists (IAL, 1982). Initially paramedical workers (PMWs) examined all patients followed by re-evaluation by experienced clinicians. The Ridley-Jopling classification was followed to confirm clinical diagnosis histopathologically in sixty six representative patients (Ridley and Jopling, 1966). The ENL and RR cases diagnosed as per the case definition mention previously (Kar and Sharma, 2010; Mohanty et al., 2007). Those cases were excluded from the study who were suffering from HIV, TB, diabetes mellitus or any other immunosuppressive disorders.

2.2.2. Healthy controls (HCs)

Unrelated healthy subjects accompanied with cases those who were staying in same locality and having similar societal and financial condition, short term project trainees of our institute were included as healthy controls. The data for knowing the socioeconomic status of participants was obtained by noting down the family income, nutritional intake and occupation mentioned in the questionnaire. Age of these controls was between 14 and 80 years of age. Exclusion criteria for healthy individuals to participate were if they have fever, viral infection, immunological diseases, and other illness and who have tuberculosis or leprosy in the past or on medication.

2.3. Selection of NOS2 SNPs

NOS2 SNPs were known to be associated with Tuberculosis, Leprosy, Malaria and other diseases in different populations (Kun et al., 2001; Jamieson et al., 2004). To the best of our knowledge *NOS2* variants were not investigated in Indian population. So, our aim was to initiate the study with these four *NOS2* SNPs (rs1800482, rs2779249, rs8078340 and rs2301369) which were evidenced to be linked with mycobacterial diseases in other population.

2.4. Genotyping of NOS2 SNPs

DNA isolation kit (Midi prep) from Qiagen, Germany was used for extraction of genomic DNA from 2 ml blood from all participants. Genotyping of *NOS2* SNPs was done by melting curve analysis on genomic DNA using fluorescence labelled hybridization probes (TIB MolBiol, Berlin, Germany) on Light Cycler 480 system (Roche Diagnostics, Berlin, Germany). The probes and primers (TIB Mol Biol, Berlin, Germany) were designed by Primer 3 software. Master mix was prepared by adding 50 ng DNA, 250 nM primers (for both forward and reverse), 250 nM probes (sensor and anchor probes) and 2.5 mM of $MgCl_2$ (Roche Diagnostics, Berlin, Germany) in a 20 μl volume of reaction mixture. At the 3′ end, the sensor probe was labelled with fluorescein (FL). At the 5′ end, the anchor probe was labelled with light cycler 640 (LC 640) dye and phosphate (PH) blocked at 3′ end. The sequences of primers and probes were mentioned in Table 2.

2.4.1. Real time PCR amplification cycles for NOS2 (rs1800482, rs2779249) genotyping

Amplification parameters: initial denaturation at 95 °C for 10 min, (ramp rate 4.4 °C/s), 30 cycles of denaturation (95 °C for