

***NRAMP1* gene polymorphism in pulmonary and spinal tuberculosis**

P. Selvaraj*, G. Chandra, S. M. Kurian, A. M. Reetha, N. Charles and P. R. Narayanan

Tuberculosis Research Centre, Indian Council of Medical Research, Chennai 600 031, India

***NRAMP1* gene polymorphisms such as 823 C/T (exon 8), deletion of TGTG in the 3'-UTR (3' untranslated region) and D543N G/A (exon 15) were studied to find out whether the gene polymorphic variants are associated with the susceptibility or resistance to tuberculosis (TB). The study was carried out in pulmonary ($n = 100$) and spinal TB patients ($n = 57$) and control subjects ($n = 112$). No difference was observed in the variant genotype frequencies of *NRAMP1* – 823 C/T, TGTG+/del and D543N G/A polymorphisms. However, a trend towards an increased frequency of the variant genotype 823 C/T (heterozygotes) was observed in pulmonary TB patients than control and spinal TB patients (odds ratio (OR): 1.6; confidence interval (CI): 0.74–3.4). A trend towards an increased frequency of the variant genotype TGTG del/del of 3'-UTR was observed in control subjects than pulmonary and spinal TB patients (OR: 0.48; CI: 0.10–1.78; OR: 0.42; CI: 0.04–2.12, respectively). The above trends were not significant. The study suggests that *NRAMP1* gene may not be associated with the susceptibility to pulmonary and spinal TB in the Indian population. Major Histocompatibility Complex (MHC) and other non-MHC gene polymorphic variants may be associated.**

In inbred mouse strains, infection with the nonpathogenic *M. bovis* BCG has been shown to be controlled by a single dominant gene present on chromosome 1, designated as *Bcg*, which is present in two allelic forms: *Bcg*^r (resistant, dominant), *Bcg*^s (susceptible, recessive)¹. The *Bcg* gene is identical to two other genes, known as *Ity*² and *Lsh*³, which control natural resistance to infection with other unrelated intracellular parasites, including *Salmonella typhimurium* and *Leishmania donovani*⁴.

A candidate gene for *Bcg* was isolated by positional cloning and was shown to be expressed in the macrophage. This gene was designated as *Nramp1*, for natural resistance-associated macrophage protein 1 (ref. 5). The *Nramp1* protein is an integral membrane protein which localizes to the phagosome membrane⁶ and may act as a divalent cation transporter. Sequence analysis of the *Nramp1* gene in *Bcg*^r and *Bcg*^s inbred strains revealed that susceptibility to infection was associated with a nonconservative glycine to aspartic acid substitution at position 169 (ref. 7).

The human homologue of the *Nramp1* gene, designated *NRAMP1*, has been suggested to be a strong candidate gene for human TB susceptibility. *NRAMP1* has been cloned and mapped to human chromosome 2q35 (refs 8 and 9) and codes for a polytopic transmembrane protein which shares a homology of 88% with mouse *Nramp1*.

Several polymorphisms have been described in the *NRAMP1* gene, and it has been suggested that they may influence the function of the gene^{10–12}. Few studies on *NRAMP1* gene polymorphism have been carried out in mycobacterial diseases such as leprosy^{13–15} and TB^{16,17}. Recently, it has been reported that genetic variants of the *NRAMP1* gene are associated with a significantly increased risk of pulmonary TB in West African (Gambia) and Korean populations^{16,17}. However, our earlier study on the *NRAMP1* CA repeat microsatellite polymorphism at 199 and 201, revealed no association with the susceptibility or resistance to pulmonary TB in the Indian population (Selvaraj *et al.*, unpublished). Since, very few studies have been carried out in TB and no such study was carried out in the Indian population, the present study was carried out to find out whether other *NRAMP1* gene variants such as 823 C/T, TGTG presence or deletion in 3'-UTR (3' untranslated region), and D543N G/A are associated with the susceptibility or resistance to pulmonary and spinal TB in south Indian population.

Subjects included in this study were 100 pulmonary TB patients, 57 spinal TB patients and 112 control subjects. Among the pulmonary TB patients, 77 were males and 23 females, aged 40.5 ± 1.3 (mean \pm SE) and 38.9 ± 2.5 years, respectively. Of the spinal TB patients, 26 were males and 31 females, aged 39.5 ± 3.3 years and 45.8 ± 2.5 years, respectively. Among the control subjects studied, 53 were males and 59 were females. The mean age with SE was 40.5 ± 1.4 year for males and 36.8 ± 1.1 year for females.

Patients attending the Tuberculosis Research Centre (TRC), Chennai with respiratory symptoms and radiographic abnormalities suggestive of pulmonary TB were studied. These patients were sputum-positive for *M. tuberculosis* by smear and culture, and are known as active pulmonary TB patients. They were given supervised short course anti TB treatment for 6 to 8 months duration and cured. They had been followed up for 5 years after treatment. At the time of blood sample collection, all the cured patients were in the quiescent stage.

Patients with clinically and radiologically active form of spinal TB involving any vertebral body from the first thoracic to the first sacral, inclusive, were studied. The pre-treatment investigations were radiograph of the chest, examination by culture of two specimens of pus from any abscess or sinus, with radiographic evidence of pulmonary TB. All these patients had received a su-

*For correspondence. (e-mail: trcicmr@md3.vsnl.net.in (imm2))

pervised short course chemotherapy of 6 to 9 months duration and followed up for a period of 5 years after treatment. At the time of blood sample collection, all these cured patients were in a quiescent stage of the disease¹⁸.

Control subjects consisted of spouses of the patients (family contacts) ($n = 65$) and staff of TRC ($n = 47$). The patient contacts (spouses) were living together with the patients before, during and after treatment and the staff of TRC, working for more than 3 years. The family contacts and the other control subjects were clinically normal at the time of blood sample collection. The patients and the contacts were not consanguineous to each other. The study subjects were randomly selected and belonged to the same ethnic origin (Indo-Dravidian descent). They were Tamil-speaking, south Indian population living in and around Chennai.

DNA was extracted from the peripheral blood white cells using a salting-out procedure¹⁹. Genotyping of 823 C/T, TGTG deletion, D543N polymorphisms of *NRAMP1* gene was carried out.

823 C/T polymorphism was defined by a point mutation at codon 249 in exon 8 (GGC to GGT) encoding glycine. The following primers were used to amplify this region¹⁰. 5'-CTT GTC CTG ACC AGG CTC CT-3'; 5'-CAT GGC TCC GAC TGA GTG AG-3'.

The PCR cycle conditions were as follows: after an initial denaturation step at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 30 s followed by 2 min extension at 72°C using 20 mM Tris, pH 8.4; 50 mM KCl; 1 mM MgCl₂; 0.4 mM dNTPs (Gibco BRL, Grand Island, NY, USA); 0.1 μM of each primer; 100 ng of genomic DNA, and 1 unit of Taq polymerase (Genei, Bangalore) in a 25 μl reaction mixture. The PCR was carried out in a programmable thermal cycler (M. J. Research Inc., Waltham, MA, USA).

The amplified 234-base pair (bp) PCR product was checked electrophoretically in a 1.5% agarose gel. Five μl of the PCR product was restriction digested with 5 units of *NarI* enzyme (Gibco BRL, Grand Island, NY, USA) at 37°C for 3 h using the manufacturer's buffer in a total volume of 20 μl. The fragments were checked in a 2% agarose gel run at 80 V for 35 min. The presence of the fragments 135 bp and 99 bp represents homozygotes of the common allele, 234 bp, 135 bp and 99 bp represent the presence of common and infrequent alleles (heterozygote carriers). Presence of only 234 bp fragment represents the homozygotes of the infrequent allele¹⁰.

TGTG presence or deletion polymorphism in the 3'-UTR was defined by a deletion of 4 bases in the 3'-UTR (55 nucleotides to the last codon in exon 15), also represented as 1729 + 55 del 4. The following primers (Gibco BRL, Grand Island, NY, USA) were used to amplify the 3'-UTR region, as described earlier^{15,16}: 5'-

GCA TCT CCC CAA TTC ATG GT-3'; 5'-AAC TGT CCC ACT CTA TCC TG-3'.

The PCR cycle conditions were as follows: after an initial denaturation step at 94°C for 4 min followed by 5 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, proceeded by 30 cycles of denaturation at 94°C for 15 s, annealing at 53°C for 30 s and extension at 72°C for 30 s followed by 2 min extension at 72°C, using 10 mM Tris, pH 8.3; 25 mM KCl; 1 mM MgCl₂; 0.4 mM dNTPs (Gibco BRL, Grand Island, NY, USA); 0.1 μM of each primer (Gibco BRL, Grand Island, NY, USA); 100 ng of genomic DNA, and 1 unit of Taq polymerase (Genei, Bangalore) in a 25 μl reaction mix¹⁵. The PCR was carried out in a programmable thermal cycler (M. J. Research Waltham, MA, USA). The amplified PCR product (240 bp) was dot-blotted and detected using the following biotinylated sequence-specific oligonucleotide probes (SSOP) (Gibco BRL, Grand Island, NY, USA). Presence of TGTG: 5'-CTG GAT GTG GAG GGG G-3'; deletion of TGTG: 5'-TGC TGG AGA GGG GGC-3'.

The PCR product (10 μl) was denatured by adding 90 μl of freshly prepared denaturing solution (9.9 ml of Tris EDTA [ethylene diamine tetraacetic acid], pH 8.0; 0.66 ml of 0.5 M EDTA, pH 8.0 and 0.88 ml of 6 M NaOH). The mixture was placed on ice for ten minutes for the denaturation process and 100 μl of 2M ammonium acetate was added to the denaturing mixture to stop the reaction. The DNA was dot-blotted on Nytran membrane (Boehringer Mannheim GmbH, Germany) using a dot-blot apparatus (Gibco BRL, Grand Island, NY, USA). The membrane was UV cross-linked in a UV cross-linker (Bio-Rad, Hercules, CA, USA) at 250 mJ and stored at -20°C until use.

The membrane was blocked with 10 ml blocking solution {4 × SSPE, 1% casein hydrolysate (SRL, Mumbai) and 0.1% laurylsarcosine (SRL, Mumbai)} in a hybridization oven (Hybaid Ltd, UK) at room temperature for 30 min. Prehybridization was done with 15 ml of hybridization solution [3M TMAC (tetramethylammonium chloride; SRL, Mumbai); 50 mM Tris, pH 8.0, 0.1% SDS (sodium dodecyl sulphate), and 2 mM EDTA] for 45 min at 53°C, in the hybridization oven.

Biotinylated SSOPs (10 pmol/ml) were added to the hybridization solution and hybridization was carried out overnight at 53°C. Membranes were then washed with 25 ml of wash buffer (2X SSPE, 0.1% SDS) at room temperature and stringency wash step was carried out with 15 ml of TMAC for 15 min at 58°C for both probes.

After stringency wash, the membrane was washed in wash buffer. The washed membrane was blocked with 20 ml of blocking solution (NaCl 125 mM, NaH₂PO₄ 17 mM, Na₂HPO₄ 8 mM, SDS 173 mM) for 15 min at room temperature. The signal was detected using Pho-

totoprobe – Star Detection Kit (New England Biolabs Inc, Beverly, USA). Blocking solution (50–60 $\mu\text{l}/\text{cm}^2$) containing streptavidin (1 $\mu\text{g}/\text{ml}$) was added to the membrane and incubated at room temperature for twenty minutes. The membrane was washed two times in 20 ml wash solution-I for 15 min each (1 : 10 diluted blocking solution, 0.5 ml/cm²) at room temperature. This was followed by biotinylated alkaline phosphatase (0.5 $\mu\text{g}/\text{ml}$ in blocking solution, 50 μl solution per cm² membrane) incubation for 20 min at room temperature. The membrane was washed twice in 20 ml of wash solution-II [Tris-HCl 10 mM, pH 9.5; NaCl 10 mM and MgCl₂ 1 mM], drained and treated with Lumigen-PPD substrate for 5–7 min. The membrane was kept wet in Saran wrap or cling film and the chemiluminescent signal was detected on X-ray film, after exposing the membrane for 5–10 min and 30 min, respectively. The membrane was stored until further use at –20°C in a wet condition to ensure that it did not dry, as drying of the membrane leads to irreversible probe and DNA hybridization.

The membranes were washed with 20 ml of stripping buffer-I (20 mM EDTA and 2X SSC) for 30 min at 65°C. The second wash was done with 20 ml of stripping buffer-II (2X SSC and 0.1% SDS) for 15 min at room temperature. The membranes were then washed in stripping buffer-III (0.2 M NaOH, 0.1% SDS) at 37°C for 30 min. The stripped membranes were re probed with the other probe.

D543N polymorphism was defined by a mutation in codon 543 involving a change of GAC (Asp) to AAC (Asn). Primers and PCR conditions were the same as those of 3'-UTR^{10,15,16}. Five μl of the PCR product (240 or 244 bp) was subjected to restriction digestion with *Ava*II enzyme (5 units) (Gibco BRL, Grand Island, NY, USA) in the presence of manufacturer's buffer in a total reaction mix of 20 μl for 3 h at 37°C. The common allele (G) was designated by the presence of 126, 79 and 39 bp bands and the infrequent allele was designated by the presence of 201 and 39 bp products¹⁰.

The frequencies of the genotypes in the patient groups and controls were analysed using χ^2 with Yates correction (χ_y^2). These analyses were carried out employing the Statcalc program (Epi Info, Version – 5; USD; Stone Mountain, GA, USA).

The *NRAMP1* – 823 C/T polymorphism did not show any deviation in the genotype frequencies between the control and the patients. However, a trend towards an increased genotype frequency of the heterozygote combination (C/T) of 823 polymorphism was seen in pulmonary TB patients than control subjects and spinal TB patients. However, this was not significant ($P > 0.05$; OR: 1.6; CI: 0.74–3.4, Table 1).

No difference in the variant genotype frequencies of 3'-UTR TGTG positive/positive (TGTG+/+) was observed among control, pulmonary and spinal TB patients. However, a trend towards an increased TGTG

positive/deletion (TGTG+/del) genotype was observed in pulmonary TB patients, but not significant ($P > 0.05$; OR: 1.4; CI: 0.6–3.4). A trend towards an increased genotype frequency of TGTG deletion/deletion (TGTG del/del) was observed in control subjects than pulmonary and spinal TB patients. However, this was not significant ($P > 0.05$; CI: 0.10–1.78; $P > 0.05$; CI: 0.04–2.12, respectively). The D543N polymorphism which is in strong linkage disequilibrium with 3'-UTR, also showed no difference among the control and the patient groups studied (Table 1).

The variant genotype frequencies of *NRAMP1* gene polymorphisms – 823 C/T (exon 8), 3'-UTR TGTG deletion and D543N (exon 15) did not differ among the control subjects and the pulmonary and spinal TB patients. Similarly, our earlier study on 5'-(CA)_n microsatellite polymorphism at exon 1, also revealed no difference in the genotype frequencies between the controls and pulmonary TB patients (Selvaraj *et al.*, unpublished). This suggests that *NRAMP1* gene may not be associated with the susceptibility to pulmonary and spinal TB in India. In a study carried out in Indian leprosy patients, no significant association was observed between the leprosy types and the four *NRAMP1* gene polymorphisms studied¹⁵.

Table 1. Genotype frequencies of *NRAMP1* – 823 C/T, 3'-UTR TGTG+/del, and D543N G/A gene polymorphisms in control, pulmonary and spinal TB patients

Variant <i>NRAMP1</i> genotype	Percentage genotype frequency		
	Control (n = 112)	Pulmonary TB (n = 100)	Spinal TB (n = 57)
823 CT			
C/C	82.1 (92)	78.0 (78)	86.0 (49)
C/T	15.2* (17)	22.0* (22)	14.0 (8)
T/T	2.7 (3)	0.0 (0)	0.0 (0)
3'-UTR			
TGTG +/+	81.3 (91)	82.0 (82)	86.0 (49)
TGTG +/del	10.7** (12)	14.0** (14)	10.5 (6)
TGTG del/del	8.0 (9)	4.0 (4)	3.5 (2)
D543N			
G/G	83.0 (93)	82.0 (82)	80.7 (46)
G/A	17.0 (19)	17.0 (17)	19.3 (11)
A/A	0.0 (0)	1.0 (1)	0.0 (0)

n = subjects studied; Numbers in parenthesis represent the subjects positive for various genotypes.

*OR = 1.4; **OR = 1.6.

The lack of association of *NRAMP1* polymorphisms in this population does not rule out an association with the gene in other ethnically different populations. In a case control study carried out in West African population, it has been shown that *NRAMP1* gene polymorphisms are associated with the susceptibility to pulmonary TB¹⁶. It has been suggested that 3'-UTR variant allele associated with susceptibility to TB is uncommon in Europeans¹⁰, but was present in about a quarter of this West African population¹⁶. Association of 3'-UTR polymorphism with the susceptibility to pulmonary TB has also been reported in Korean population¹⁷.

Our earlier study²⁰ on HLA revealed the association of HLA-DR2 with the susceptibility to pulmonary TB, whereas no such association was observed in West African population. Further, our study²¹ on mannose-binding protein (MBP) gene polymorphism revealed that functional mutant homozygotes are associated with the susceptibility to pulmonary TB. Whereas in Gambian pulmonary TB patients and control, neither homozygotes nor heterozygotes of mannose-binding lectin (MBL) gene variants 54 and 57 (functional mutant homozygotes) were at increased risk of pulmonary TB²². Moreover, our study on vitamin-D receptor gene polymorphism²³ revealed the association of tt genotype with the susceptibility to pulmonary TB in female patients, and TT genotype with resistance to pulmonary TB in female contacts in India. In the Gambian pulmonary TB patients, the tt genotype of *VDR* gene was found less frequently in cases of pulmonary TB, suggesting that this genotype may be associated with resistance to pulmonary TB²⁴. Interestingly, *NRAMP1* gene polymorphic variants are associated with the susceptibility to TB in the Gambian population (West Africa)¹⁶, whereas our present study in Indian TB patients revealed no association of *NRAMP1* gene variants with the susceptibility or resistance to TB. This type of association may be due to gene-environment interaction.

The present study suggests that *NRAMP1* gene polymorphic variants may not be associated with the susceptibility to TB similar to leprosy (another mycobacterial disease). In the Indian population, *NRAMP1* gene variants may not be the dominant alleles, probably other Major Histocompatibility Complex (MHC) and non-MHC genes may be associated with the susceptibility to mycobacterial diseases such as TB and leprosy.

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ACKNOWLEDGEMENTS. G.C. and S.M.K. were financially supported in the form of Junior and Senior Research fellowships by University Grants Commission and Council of Scientific and Industrial Research, New Delhi, respectively.

Received 1 October 2001; revised accepted 7 December 2001