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Original Research Article

Polymorphism in the NRAMP1 gene as a risk factor for pulmonary tuberculosis -A case control study

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ABSTRACT

Objective: Polymorphism in this gene was determined in pulmonary tuberculosis patients in order to explore this association.
Design: We assigned 34 patients with active pulmonary tuberculosis and 30 subjects with latent tuberculosis to the infected group, and 30 healthy household cases to control group. Three polymorphisms were studied, a non-conservative single-base substitution at codon 543(D543N), a single nucleotide change in intron 4(469_14G/C, INT4) and TGTG deletion in the 3 ' untranslated region (1729_55del4, 3 ' UTR).
Results: No polymorphism was detected in the later two regions. G/A variant was more commonly found in the uninfected group with G/G variant in the infected group. Among the infected group, the G/A variant was significantly associated (44.11%) with active pulmonary tuberculosis. Although homozygous variants (A/A) were also associated (20.58%) with active disease, the prevalence of heterozygous variant found to have a risk eleven folds higher than the later (OR= 11.5).
Conclusion: The heterozygous genotype seemed to affect the burden of M. tuberculosis, and in particular the clinical presentation of the disease.
Setting: Defective production of protein Nramp1, encoded by gene NRAMP1 disturbs the normal innate host resistance.

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1. Introduction

It is estimated that 10.0 million people (range, 9.0–11.1 million) developed TB disease in 2017 globally.[1] No country or age groups were spared. However adults comprised 90% (aged $\geq$ 15 years), 9% were with HIV (72% in Africa). India alone documented 27% of total.[2] The progression of the disease occurs in a multitude of directions under the influence of host immune response, the efficacy of which is related to the various intrinsic factors. These intrinsic factors comprise of the genetic make-up of the host immune system and extrinsic factors such as insult to the immune system or nutritional and physiological state of the host.[3] The Natural Resistance Associated Macrophage Protein 1, designated NRAMP1, encoded by SLC11A1 gene is a strong candidate gene for human tuberculosis with its multiple effects on macrophage activation and host innate immune response to infections.[4] NRAMP1 is located on the long arm of chromosome-2 (2q35).[5] Meta-analytical studies until September 2010 indicated towards an ethnicity specific effect of SLC11A1 polymorphisms on tuberculosis risk.[6] Although several functionally significant polymorphisms have been ascribed to the NRAMP1 gene, few studies have been conducted with respect to mycobacterial diseases such as Tuberculosis.[7] Through various studies worldwide, it has been proposed that there is a significant relationship of NRAMP1 polymorphism to Tuberculosis progression since NRAMP1 is a membrane protein expressed on the macrophage surface playing a role in immune responses by phagocytosis and antigen presentation.[8] Various studies around the world found close relation between genetic polymorphisms such as the G/C single nucleotide change in intron 4(469_14G/C), a non-conservative single-base

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substitution at codon 543 that changes aspartic acid (Asp) to asparagine (Asn), and TGTG deletion in the 3' untranslated region and mycobacterial diseases.[5,9–11]

This study was aimed at investigating the association between NRAMP1 polymorphisms and pulmonary tuberculosis by determining the incidence of NRAMP1 genetic polymorphisms among patients with pulmonary tuberculosis disease, latent tuberculosis and healthy household adults.

2. Materials and Methods

2.1. Study population

During the study period from 1st July, 2016 to 31st June, 2017, total of 43 new smear positive sputum samples from patients (15-70yrs.) coming to the outpatient department for tuberculosis screening were sent to the Microbiology Department of Assam Medical College for culture and sensitivity. Subjects sensitive to first line anti-tubercular drugs were included in the study. A total of 34 new smear positive cases who consented for the study were included in the case group (n=34). During the same period, among those sent to the department for Mantoux test, 30 with positive result and consenting to the study were included in the latent infection group (n=30). The healthy household contacts were selected from among the family members and close asymptomatic contacts of pulmonary tuberculosis patients who were tested negative for M. tuberculosis infection and consented to the study (n=30). Thusa total of 94 subjects with age more than 15 years and of both sexes, matching the inclusion criteria and without any conditions that could induce changes in acquired immunity (diabetes mellitus, chronic liver disease, chronic alcohol ingestion, gastrectomy, malnutrition, pregnancy, malignancy, history of immunosuppressant use, organ transplant, Human Immunodeficiency Virus [HIV] infection) were included in the study. All demographic, socioeconomic, clinical, morbidity data and relevant investigations collected through a pre-prepared study schedule. All subjects were Indian and not enrolled in any previous study.

2.2. Sample collection and extraction of genomic DNA

2 ml. of blood was drawn aseptically by venipuncture from cubital vein and collected under aseptic conditions in EDTA vial. 1ml. was used to determine complete blood count and rest stored at -80ºC after collection. 200μl of each blood sample was used for extraction of DNA by QIAamp DNA Mini Kit (according to kit manufacturer's protocol).[12] Integrity of the extracted DNA was checked by running 5μl of DNA on 0.8% agarose gel stained with Ethidium Bromide in microplate spectrophotometer Multiskan GO from Thermo Scientific. Samples showing 260/280 ratio between 1.8 and 2 were included for downstream application.

2.3. NRAMP1 genotyping

The polymorphisms typed were

1. Single-nucleotide change in intron 4 (INT4) (469+14G/C)
2. D543N, a non-conservative single-base substitution at codon 543, which changes aspartic acid to asparagine; and
3. 3′UTR, a TGTG deletion in the 3′untranslated region (1729+55del4).

Genotyping was done by using Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphism (RFLP)

2.4. Polymerasechain reaction

The conventional PCR was carried out according to previously published literature after confirmation of primers, standardization of the process through gradient method required modifications. The primer sequences were confirmed and proof read for the particular purpose by performing a blast search prior to the study. Gradient PCR is a technique that allows the empirical determination of an optimal annealing temperature using the least number of steps. The primers used to amplify each polymorphism were as follows: for the INT4 5′CTCTGGCTGAAGGCTCTCC3' and 5′TGTGCTATCAGTTGAGCCTC3', for D543N and 3 ' UTR, 5′GCATCTCCCCAATTCATGGT3′ and 5′AACTGTCCCACTCTATCCTG3′, (Integrated DNA Technologies, USA). For INT4 and D543N, composition for a 20μL solution used in PCR contained DNA 2μ L, forward and reverse primers 1μL each, nuclease free water 6μL and PCR Master Mix (Promega Corporation) 10μL which is a premixed, ready-to-use solution containing *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations. For 3'UTR, the composition for a 40 μL solution contained DNA 2μL, forward and reverse primers 1μL each, nuclease free water 1 6μL and PCR Master Mix (Promega Corporation) 20μL. The Arktik Thermal Cycler (Thermo Fisher Scientific, Inc., Waltham MA) was programmed to run separately all three cycles. For INT4, the reaction was allowed to continue for 5 min at 94 ° C, denaturation for 1 min at 94 ° C, annealing for 1 min at 56 ° C, extension for 1 min at 72 °C repeated for 30 cycles, and 5 min at 72 ° C, then stored at 4 ° C. With D543N and 3' UTR, denaturation was done at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec and 7 min at 72 °C. A ll other procedures were identical. After PCR, electrophoresis was conducted using 2% agarose gel.

The PCR end products were analysed by electrophoresis on 3% agarose gel stained with ethidium bromide at 90V for 30min. and the bands were visualized using a using a Gel

Documentation System (Gel Doc XR+, Molecular Imager Gel Documentation System from Bio-Rad Laboratories (India) Private Limited). After ethidium bromide staining, 623 base pairs (bp) of DNA fragment for INT4, and 244 bp fragment for D543N and 3 ' UTR were detected.(Figure 1)

2.5. Restriction fragment length Polymorphism (RFLP)

Base sequence of wild type and mutant type was detected by PCR-RFLP with the help of restriction enzymes. For INT4, *ApaI* (GGGCC_C) was used. Incase of G → C mutant type, verification of two bands of 455 bp and 169 bp was done; For D543N, *AvaII* (G_GWCC) was used, with verification of three bands of 126 bp, 79 bp and 39 bp for allele G(Asp) and two bands of 201 bp and 39 bp for allele A(Asn). For 3 ' UTR, *FokI* {GGATG(9/13)} was used with allele TGTG verified by 211 bp and 33 bp fragments and del by a 240 bp fragment. For *ApaI* and *AvaII,*the solution contained respective restriction endonuclease 2μL, buffer (100 mM Tris-HCl [pH 7.5], 100 mM MgCl2, 10 mM dithiothreitol) 3μL, substrate DNA 5μL. For *FokI* ,the solution contained *FokI* 0.5μL, buffer 5μL and substrate DNA 30μL. The reaction solution for ApaI and AvaII incubated for 2hrs. and FokI for 5min. at 37 °C. Verification of the fragment size was done through electrophoresis. (Figure 2)

2.6. Statistical analysis

Statistical analysis was done using the Microsoft Excel, 2007, Epi Info 7 and online software like Social Science Statistics and Graph Pad Version 7.0.1. Non parametric tests were done as and when necessary. Tests were considered to be significant if p value was <0.05. The associations between the allelic/genotypic frequencies and the clinical state of subjects included in the study, as well as the odds ratio (OR) for the susceptibility to infection, were obtained by Chi-square test.

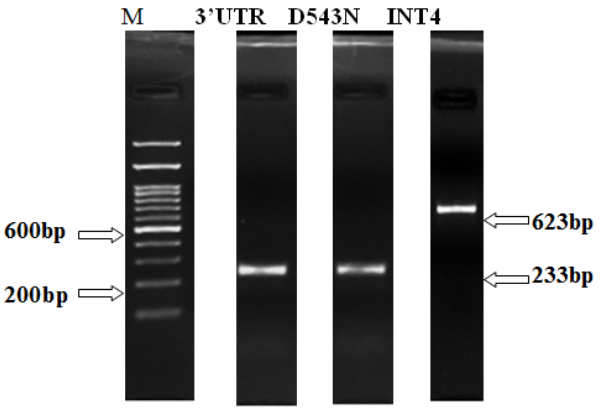

Fig. 1: Agarose Gel Electrophoresis of PCR products.

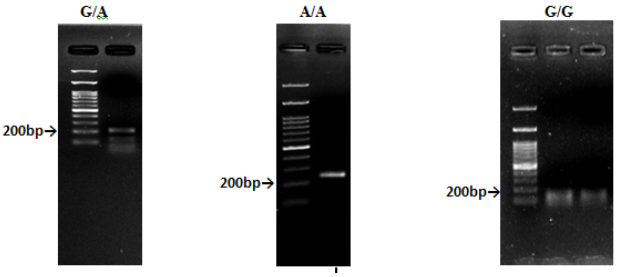

Fig. 2: Agarose gel electrophoresis of PCR-RFLP products of D543N.

3. Results

Preponderance of tuberculosis among males was found to be higher. The ratio of male to female for Active TB was 23:11, Latent TB 17:13 but household contacts 13:17. This can be attributed to the care-giving attitude of the female family members.

Polymorphic variants were observed in D543N loci and only wild type variants were observed in INT4 and 3'UTR loci. The frequencies of D543N polymorphism in *NRAMP1* were G/G: G/A: A/A = 35.29%: 44.12%: 20.5% for the Pulmonary tuberculosis group, 43.33%: 6%: 50% in the latent tuberculosis group and 6%: 66.6%: 26.6% in the household contact group.

3.1. Association of mutation with smear positive pulmonary tuberculosis

Comparison was done between wild and mutant type in the pulmonary tuberculosis case group and latent tubercular contact group, aimed at finding whether NRAMP1 mutation at D543N reg ion is associated with risk of developing severe clinical tuberculosis. The heterozygous G/A variants were identified in the D543N loci in 44.11% with the microscopy-positive form of pulmonary tuberculosis, whereas homozygous A/A variants were exhibited in 20.58% of subjects. The heterozygous mutant genotype G/A was found to have significant association with the development of severe tubercular disease with OR=11.5 and A/A showed significant association in the control group (*p-value* <0.5). However, a low OR=0.26 value of the variant A/A suggests a weaker association with development of microscopy positive severe tuberculosis. Wild type G/G might have some protective role with a low OR of 0.71. (Table 1)

3.2. Association of mutation with tubercular infection

Comparison of wild type and mutant type in the case group who acquired the infection, comprising of pulmonary and latent tuberculosis positive subjects with control group comprising of household contacts showed the wild type variant G/G significantly present in subjects infected with

Table 1: Comparison of wild and mutant type between case and latent control group.

Polymorphism D543N	No. of subjectsPulmonary tuberculosis n%	No. of subjectsLatent Tuberculosis n%	OR	95% confidence interval	p-value
G/GG/A A/A	12 15 7	13 2 15	0.71 11.5 0.26	0.26-1.95 2.26-54 0.08-0.77	0.51 <0.05 <0.05

Table 2: Comparison of wild and mutant type between infected and non-infected group.

Polymorphism D643N	No. of subjectsInfectedn% n=64	No. of subjectsHousehold contactsn% n=30	OR	95% confidence interval	p-value
G/G G/A A/A	25 17 22	2 20 8	8.97 0.18 1.44	1.96-41.02 0.07-0.46 0.55-3.76	<0.05 <0.05 0.45

M. tuberculosis. (p <0.05) whereas G/A present in household contacts. This analysis was aimed at finding whether NRAMP1 mutation affected susceptibility to infection by tubercle bacilli. The prevalence of G/A mutant genotype is found to be lower in patients who had acquired the infection as compared to subjects who were exposed to the disease but had not been infected (*p-value* <0.05). The heterozygous mutant G/A was associated with household contacts and perhaps exerts protection against the disease. However, the G/G genotype showed a high OR suggesting that the wild genotype might not have any protective role against tubercular infection. (Table 2).

4. Discussion

The polytopic integral membrane protein NRAMP1 is localised to the late endosomal and lysosomal compartments[13–15] and functions as a metal ion transporter regulating,[16] and regulated by, cellular iron levels.[14] Various studies around the globe have shown that a genetic background exerts a crucial role on the possible influence of the NRAMP1 gene in susceptibility to infection by the tubercle bacilli. In this regard, it has been reported that the protein encoded by NRAMP1/SLC11A1, that is Nramp1, seems to have a role in the control of bacilli growth and disease progression rather than in susceptibility to tubercular infection.[17,18] A similar study by P. Selvaraj in Southern India suggested that NRAMP1 gene may not be associated with the susceptibility to pulmonary tuberculosis.[7] However study conducted in China by Wenghong Zhang[17] and Julio C. Delgado[19] in Cambodia showed positive effect of D543N mutation on tubercular susceptibility. The later also showed positive relationship of 3'UTR which is in disparity to our present study. But, this can be explained by the study where it was suggested by B.C. Dodd that Tai people and Mongolians share a recent common origin.[20] In fact, the D543N variant allele can be detected in ~20% of the Chinese population, as in alignment with the present study. The INT4 variant allele can be found in ~50% of white Europeans, whereas the frequency of this allele is as low as 20% in the Chinese population.[11,21] Our study population comprised of consecutive cases reporting within the definite one year study duration. Hence, few subjects pertaining to other communities but, nevertheless, hailing from the same region have also been included. Therefore, it involves a regional group of Upper Assam, with a majority of indigenous subjects. Moreover, a number of factors may be responsible for the discrepant results reported by the extended studies stretched across the globe. Ethnic or racial backgrounds of the study populations can certainly introduce some variation.[21]

4.1. Association of NRAMP1-D543N polymorphism with active pulmonary tuberculosis and latently infected tuberculosis

The mutant variant, heterozygous G/A was significant ly associated with the development of active tubercular disease (44.11%, p-value <0.05) whereas homozygous A/A variant was present in 20.58% of active pulmonary tuberculosis subjects (p-value <0.05). This finding is similar to that of Richard Bellamy in West Africa[22] and Wenghong Zhang in China.[17]

In support of this, Zhang suggested a possibility that incomplete linkage disequilibrium between the typed polymorphisms and an unknown mutation affecting function might have obscured the true dominance pattern of the resistance and susceptibility genotypes. Alternatively, there is also a possibility that the heterozygous genotype (G/A) is able to initiate an increase in the divalent cation concentration in the phagolysosome of the human host hence providing a more favourable condition for mycobacterial growth. However, apart from the above hypotheses, a further dire prospect maybe that the NRAMP1 variant homozygotes, already infected with M. tuberculosis in their childhood, surrender to the pathogen rapidly, which might explain their fewer representation among adults and the considered case group.

4.2. Association of NRAMP1-D543N mutation with Mycobacterium tuberculosis infection

In this study the heterozygous mutant G/A was more commonly found in the non infected group ($p < 0.05$) whereas no relation was seen with the A/A variant between infected and non-infected group. ($p = 0.45$). The infected group composed of cases with pulmonary tuberculosis and latent infection as compared to healthy household contacts. This is similar to the Indian study conducted by P. Selvaraj, who also found G/A more commonly present in non-infected group whereas A/A did not show any association.[7] However, studies on NRAMP1 polymorphism affecting tubercular susceptibility from around other Asian countries like Indonesia show varied results.[23–25] This is important as similar to the vast Indian genetic diversity, Indonesian archipelago also stretches from west to east between the continent Asia and Australia and harbours many ethnicities. Hence further regional studies are hoped for.

The present study also shows that G/G, as wild-type allele, were more frequently found in the infected study group. ($p<0.05$, OR=8.97). This is similar to the findings of Trevino A. Pakasi in his study from Kupang, Indonesia.[23] These results therefore demonstrate that NRAMP1-D543N polymorphic variants G/A and A/A are not associated, whereas G/G is associated with susceptibility to *M.tuberculosis* infection among patients in areas of Assam where tuberculosis is endemic. The wild type variant, therefore, presumably does not confer any protective effect against the organism.

5. Conclusion

A significant genotypic association was observed between NRAMP1-D543N polymorphism and pulmonary tuberculosis. The heterozygous genotype (G/A) seemed to affect the burden of *M. tuberculosis*, and in particular the clinical presentation of the disease.

6. Source of Funding

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7. Conflict of Interest

None.

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