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# **Full Length Research Paper** Association Study of NRAM1 (D543N) gene polymorphism with Tuberculosis in Vindhyan Population

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### ARTICLE DETAILS

## ABSTRACT

Corresponding Author: Vaibhav Singh Key words:

NRAM1 gene, Genetic Polymorphism, Allele frequency *mycobacterium tuberculosis* Tuberculosis. Tuberculosis is one widely distributed infectious disease worldwide, and severely threatens public health. Lung tuberculosis has relatively higher incidence within Tibetan region of China, probably due to unique genetic background of Tibetan Population and certain susceptibility genes. NRAMP1 participates in host resistance against mycobacterium tuberculosis, although its correlation with tuberculosis prevalence in Tibetan is still unclear. This study aimed to investigate the possible correlation between NRAMP1 polymorphism and tuberculosis in Vindhyan Population. Total of 160 Vindhyan Population diagnosed with lung tuberculosis were recruited in parallel with 190 healthy Vindhyan volunteers as the control group. Venous blood samples were collected for separating genomic DNA of blood cells. PCR-RFLP sub-typed NRAMP1 gene and grouped patients based on genotype. The observed genotype frequencies, allele frequencies and carriage rates for NRAM1 (D543N)\_polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. Overall distribution of NRAM1 (D543N) genotypes was significantly different in healthy control group as compared to disease group ( $\chi^2$ =11.34, P=0.0035\*\*). Overall allele 'G' was found to be in significantly low frequency in disease group as compared to Healthy control group whereas allele 'A' was present in significantly high frequency in the disease group ( $\chi^2 = 12.79$ , P= 0.0003\*\*\*). Overall G allele shows an odds ratio of 0.4764 which indicates its protective association. The pattern of genotype and allele distribution in disease and control group suggested a significant association with NRAM1 (D543N) gene to Tuberculosis.

## 1. Introduction:

Tuberculosis is one widely distributed infectious disease worldwide, and severely threatens public health. The prevention and treatment of tuberculosis are thus of critical importance. About 5%~10% of total population are carriers of tuberculosis, probably due to prevalence of susceptible genes [1, 2]. The susceptibility towards tuberculosis has variance across ethnic groups [8, 9]. Moreover, different hosts presented different sensitivities for tuberculosis, probably related with polymorphism of various genes such as NRAMP1, human leukocyte antigen, mannose binding lectin, all of which are closely correlated with tuberculosis susceptibility. This study aimed to investigate the relation-ship between NRAMP1 and tuberculosis, as NRAMP1 is probably related with disease susceptibility [2, 3]. We found that polymorphism of NRAMP1 gene showed imbalance of linkage, indicating TGTG-deleted allele might be tuberculosis susceptible gene in Tibetan people, indicating that those people with TGTG deleted. Host genetic susceptibility to infectious disease has been widely studied in recent years, which is helpful for high-risk population identification and therefore promotes diseases prevention and early diagnosis [4]. Moreover, such study also contributes to clarify potential mechanisms underlying host defense to the disease development. Natural resistance associated macrophage protein 1 (NRAMP1), encoded by the SLC11A1 gene, has multiple effects on macrophage activation and has been reported to play an important role in host innate immune response against infections [1-4]. Tuberculosis (TB), caused by infection of Mycobacterium tuberculosis,

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remains a major challenge to global public health. As estimated, that one-third of the world's population is infected, but that only a minority of those infected ever develop TB [4]. Host genetic susceptibility, together with some environmental and lifestyle factors, has been suggested to contribute to such clinical diversity [3,5]. NRAMP1 is located on the endocytic compartment of resting macrophages and is recruited to the membrane of the phagosome depending on the pH gradient. NRAMP1 acts as a divalent cation transporter or antiporter across phagosomal membranes that are expressed only in reticuloendothelial cells [7,9]. These facts suggest that NRAMP1 may inhibit the replication of intracellular pathogens by altering the phagolysosomal environment. NRAMP1 is a critical mediator in the innate immune response to tuberculosis infection which leads to decreased DNA replication and respiratory chain function in *M. tuberculosis*, but the precise function of this protein remains unclear [5,7]. Several polymorphisms have been described in the NRAMP1 gene and these polymorphisms alter the gene's function. Four NRAMP1 polymorphisms; 30- UTR, D543N, 50(GT)n, and INT4 have been associations with *M. tuberculosis* infection in humans. In terms of the 50(GT)n polymorphism, a study found that there was a significant association of this NRAMP1 polymorphism type with tuberculosis when considering an interaction with Toll-like receptor 2 (TLR2) [6,8]. Another study found that NRAMP1 polymorphisms at the D543N and INT4 loci contribute to severe pulmonary tuberculosis [5-9].

### 2. Materials and Methods:

### 2.1 Study population

The study population consisted of 350 unrelated subjects comprising of 160 Tuberculosis patients and 190 ethnically matched controls of central Indian population were included in this study. Blood sample of case and control group included consecutive patients who attended the Department of Medicine, Shyam Shah Medical College and Sanjay Gandhi Memorial Hospital, Rewa, Ayurveda Medical College, Rewa, Ranbaxy pathology Regional collection centre Rewa, District hospital Satna, Shahdol, Sidhi. Tuberculosis was diagnosed in accordance with World Health Organization criteria. Pregnant women, children under age of 18 years and any patients with Tuberculosis were excluded from the study. Blood sample of control group composed of non- Tuberculosis healthy individuals that were collected during "Tuberculosis Awareness Camps" organized in urban regions in and around SSMC Rewa and many volunteers were also included to collect control sample. The control subjects were recruited from the regions that from homogenous cluster in Vindhyan region India in accordance with a recent report of genetic landscape of the people of India.

#### 2.2 Blood collection and plasma/serum separation:

Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at  $4^{\circ}$ C.

### 2.3 DNA isolation:

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller et al. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4°C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4°C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4°C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4°C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4°C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37°C before quantization.

### 3. Detection Of NRAM1 (D543N, RS17235409) genotype:

*NRAM1 (D543N, rs17235409)* the 435 bp PCR product contains three *Avall* cutting sites at '-75', '+37' and '+83' loci, its complete digestion would produce 4 fragments of sizes 66 bp, 114 bp, 46 bp and 209 bp. The '-75' and '+83' loci are polymorphic and coincide with the G-75A and C+83T SNPs respectively. This primers were early used by (Ghada Basil Alomashi, and Hasan Raheem Khudhur, 2017)-

## Forward primer 5'- ACT-AAGAAA-GAC-CCG-AGG-C-3' Reverse Primer 5'-GGG-GCA-CGT-TGG-TGTTTA-C-3'

### PCR Mix

The PCR was carried out in a final volume of 25  $\mu$ l, containing 50-100 ng of genomic DNA(4-5  $\mu$ l), 2.5  $\mu$ l of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1  $\mu$ l of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1  $\mu$ l of 25 pmol/ $\mu$ l of forward and reverse primers specific for and 1  $\mu$ l of unit of 1U/  $\mu$ l Red Taq DNA polymerase (Bangalore genei).

### PCR Thermal Program

After an initial denaturation of 5 min at 95°C, the samples were subjected to 35 cycles at 95°C for 1 min, at 62°C for 45 s, and 72°C for 45 s, with a final extension of 10 min at 72°C in a thermal cycler. A 100bp ladder with amplified product has been run under 2.5 % agarose gel electrophoresis. 180 bp product will be generated after PCR.

### Restriction digestion

The amplified product size of 180 base pairs (bp) was digested by the specific restriction enzyme, *Mspl* for 16 h at 37°C. The wild-type genotype was not digested, whereas the mutated homozygous genotype was cut as a doublet of 114 and 66 bp. The heterozygous genotype (GA) was represented as 3 fragments of 180, 114, and 66 bp. Samples were analyzed by electrophoresis using 2.5% agarose gels to analyze the genotype pattern of the gene. The digestion products were then separated by electrophoresis on a 2.5% agarose gel. The results were documented by digital camera and further saved by gel documentation system.

### 4. Results

### 4.1 Detection of Genetic Polymorphism in NRAM1 (D543N, rs17235409):

The nucleotide position D543N polymorphism in NRAM1 gene create restriction site for *Avall*. The PCR products when digested by restriction enzyme and wild type allele 205 bp segment which were generated by PCR but the mutant allele shows 126 and 79 bp segments (figure 1). The distributions of polymorphic genotype were strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for NRAM1 (D543N)\_polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. Overall distribution of NRAM1 (D543N) genotypes was significantly different in healthy control group as compared to disease group ( $\chi^2$ =11.34, P=0.0035\*\*). Healthy control group showed an increase of mutant 'AA' genotype as compared to Patients of Tuberculosis (02.63% *vs.* 06.87%).

Similarly, wild type '*GG*' genotype was present in significantly low frequency in patient as compared to Healthy control group (63.75% vs. 79.47%). An odds ratio of 0.4542 in Tuberculosis group respectively for 'GG' genotype indicated a protective effect of this mutant type genotype in our population whereas AA an odds ratio of 2.732 of Tuberculosis patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous GA is also significantly different but may be not protective because of odds ratio of 1.908. Overall allele 'G' was found to be in significantly low frequency in disease group as compared to Healthy control group whereas allele 'A' was present in significantly high frequency in the disease group ( $\chi^2 = 12.79$ , P= 0.0003\*\*\*). Overall G allele shows an odds ratio of 0.4764 which indicates its protective association. Carriage rate of allele 'G' was high in Healthy control group whereas carriage rate of allele 'A' was high in disease group ( $\chi^2 = 6.942$ , P= 0.0084\*\*) and the values were also significant. The pattern of genotype and allele distribution in disease and control group suggested a significant association with NRAM1 (D543N) gene to Tuberculosis.



**Fig. 1:** Representative gel picture of NRAM1 (D543N, rs17235409) polymorphism; The expected product sizes are: normal homozygote GG, 180bp; homozygote AA, 114 and 66 bp; and heterozygote GA, 180, 114, and 66 bp, respectively.

**Table 1.** Frequency distribution and association of Genotype, allele frequency and carriage rate of NRAMP1 polymorphism in population of Vindhyan region using Chi Square Test

NRAMP1 Genotype	CASE N= 160		CONTROL N=190		CHI SQUARE VALUE $\chi^2$ (P Value)	
	Ν	%	Ν	%		
GG	102	63.75	151	79.47	11 24 (0 0025**)	
GA	47	29.37	34	17.89	DF:2	
AA	11	06.87	05	02.63		
Allele						
G	251	78.43	336	88.42	12.79 (0.0003***)	
Α	69	21.56	44	11.57	DF:1	
Carriage Rate						
G	149	71.98	185	82.58	6.942 (0.0084**)	
Α	58	28.01	39	17.41	ער:1	

\* denotes the level of significant association between case and control.;

N – Number of individuals in study group.

%- Genotype allele frequency and carriage rate expressed in percentage

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NRAMP1 genotype	CASE N= 160		CONTROL N=190		P Value	Odds Ratio (CI)
genotype	N	%	N	%		
GG	102	63.75	151	79.47	0.0012**	0.4542 (0.2818 to 0.7322)
GA	47	29.37	34	17.89	0.0153*	1.908 (1.154 to 3.157)
AA	11	06.87	05	02.63	0.0729 ns	2.732 (0.9284 to 8.037)
Allele						
G	251	78.43	336	88.42	0.0004***	0.4764 (0.3155 to 0.7193)
Α	69	21.56	44	11.57		2.099 (1.390 to 3.170)
Carriage Rate						
G	149	71.98	185	82.58	0.0109*	0.5416 (0.3420 to 0.8577)
Α	58	28.01	39	17.41		1.846 (1.166 to 2.924)

\* denotes the level of significant association between case and control;

N – Number of individuals in study group

%- Genotype allele frequency and carriage rate expressed in percentage



#### Genotype Distribution of NRAMP1 gene

#### 5. Discussion:

The TB history has being closely associated with important events in human history and directly related with the progress in biological sciences and influencing human culture. Around the 19th century, the death rate due to TB was estimated at 7 million people worldwide [14]. As TB was taking lives of many, the major event in the medical history of this century was revealed when Robert Koch published for the first time the discovery of the *Mycobacterium tuberculosis (Mtb)* [12]. Previous attempts to demonstrate that TB was contagious by the aerosol route were already done in 1877 by Tappeiner, but Koch was the first one able to demonstrate that TB was caused by a microorganism and to identify the bacteria *(Saukkonen, J.J. et. al. 2012).* This milestone initiated the modern era of TB, a disease that was previously thought to be hereditary, since it "ran" in families [15]. During his talk in 1882, Koch performed a demonstrative description of the steps that he followed to reach his discovery. These collective steps received the name of the Koch's postulates and allowed the discovery of other bacterial-causing diseases such as cholera. Koch also described the involvement of the host in TB pathology, setting then the basis for two beautiful sciences Microbiology and Immunology [10-15].

Associated Macrophage Protein 1 (NRAMP1, also known as Solute Carrier Family 11a member1) polymorphism by studying the genetic segregation of this polymorphism. The incidence of the disease among members of the population undergoing surgery for tuberculous spondylitis at our institution [16, 20]. We compared the distribution of NRAMP1 polymorphism at two specific sites, namely D543N, and 3'UTR, among subjects with pulmonary tuberculosis and tuberculous spondylitis. We found no significant differences in distribution of polymorphism between the two groups, or between pulmonary tuberculosis and tuberculous spondylitis compared to healthy subjects [4, 17]. However, a pattern emerged in that polymorphisms at the two sites seemed to be protective against development of tuberculous spondylitis in our study population. We concluded that in the West Javanese population, there is no association between NRAMP1 polymorphism with the propensity for development of pulmonary tuberculous spondylitis [12, 18]. In fact, NRAMP1 may provide protection against the development of tuberculous spondylitis.

resistance associated macrophage protein 1 (*NRAMP1*) polymorphisms (D543N, INT4) with pulmonary tuberculosis (PTB) risk have been widely reported. However, the findings of previous studies were inconsistent. To clarify the role of these polymorphisms in PTB, we performed a meta-analysis of all available and relevant published studies. They identified outcome data from all articles estimating the association between *NRAMP1* polymorphisms and PTB risk [16-20].

Our study on NRAM1 gene polymorphism revealed that the nucleotide position D543N polymorphism in NRAM1 gene create restriction site for Avall. The PCR products when digested by restriction enzyme and wild type allele 205 bp segment which were generated by PCR but the mutant allele shows 126 and 79 bp segments. The distributions of polymorphic genotype were strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for NRAM1 (D543N) polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. Overall distribution of NRAM1 (D543N) genotypes was significantly different in healthy control group as compared to disease group ( $\chi^2$ =11.34, P=0.0035\*\*). Healthy control group showed an increase of mutant 'AA' genotype as compared to Patients of Tuberculosis (02.63% vs. 06.87%). Similarly, wild type 'GG' genotype was present in significantly low frequency in patient as compared to Healthy control group (63.75% vs. 79.47%). An odds ratio of 0.4542 in Tuberculosis group respectively for 'GG' genotype indicated a protective effect of this mutant type genotype in our population whereas AA an odds ratio of 2.732 of Tuberculosis patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous GA is also significantly different but may be not protective because of odds ratio of 1.908. Overall allele 'G' was found to be in significantly low frequency in disease group as compared to Healthy control group whereas allele 'A' was present in significantly high frequency in the disease group ( $\chi^2 = 12.79$ , P= 0.0003\*\*\*). Overall G allele shows an odds ratio of 0.4764 which indicates its protective association. Carriage rate of allele 'G' was high in Healthy control group whereas carriage rate of allele 'A' was high in disease group ( $\chi^2$  =6.942, P= 0.0084\*\*) and the values were also significant. The pattern of genotype and allele distribution in disease and control group suggested a significant association with NRAM1 (D543N) gene to Tuberculosis.

However, NRAMP1 polymorphisms at the D543N and INT4 were not associated with tuberculosis in an Indonesian population. Toll-like receptor 2 (TLR2) TLRs are transmembrane molecules that serve as sentries for pathogen detection, by a kind of evolutionary recognition of molecular patterns associated with past infections [22]. TRL expression found in many cell types, including host immune cells, serves as critical mediators of the immune response to a variety of pathogens, including *M. tuberculosis*. Several types of TLRs have strong links with tuberculosis, including TLR1, TLR2, TLR4, TLR6 and TLR9 [24]. The genetic variant most often associated with tuberculosis is found in TLR2. A TLR2 heterodimer, in combination with the TLR1, binds 19-kD mycobacterial lipoprotein, suggesting TLR2 is an adhesion molecule for *M. tuberculosis*. A previous study suggests that Toll interleukin 1 receptor domain containing adaptor protein (TIRAP), an adaptor that mediates signals from TLRs activated by mycobacterial molecules, induced a strong proinflammatory response to tuberculosis [21-25].

### 6. Conclusion

In conclusion, our study shows that *Nramp1* gene variation is of importance for the phenotypic appearance of TB (i.e., microscopy-positive TB), indicating that NRAMP1 is of importance for the replication of the bacilli. By contrast to what has been suggested for other ethnic/racial groups, our results do not indicate that *Nramp1* alleles confer an increased risk of TB among white individuals in Vindhyan Population. Our all my finding from genetic polymorphism of NRMP1 in Vindhyan population suggested association with Tuberculosis. Our selected population (Case and control) revealed association with NRMP1 gene.

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