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Association study of SLC11A1 gene (3'UTR) gene polymorphism with pediatric tuberculosis in Madhya Pradesh, India

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Abstract

Natural resistance associated macrophage protein 1 (NRAMP1) encoded by the SLC11A1 quality, has been depicted to manage macrophage initiation and be related with irresistible and immune system ailments. The connection between SLC11A1 polymorphisms and tuberculosis vulnerability has been considered in various populaces. these are no relationship between NRAMP1 polymorphism with the affinity for improvement of pneumonic tuberculosis or tuberculous spondylitis. Truth be told, NRAMP1 may give insurance against the improvement of tuberculous spondylitis. The human homologue of the NRAMP1, assigned NRAMP1, has been cloned and mapped to chromosome 2q35. In ongoing occasions, number of qualities has been explored in different case control thinks about, of which the regular resistance– related macrophage protein (NRAMP1) is believed to be imperative in the intracellular slaughtering of Mycobacteria.

In our populace, (NRAMP1) isn't related with pediatric tuberculosis. TGTG +/+ genotype did not demonstrate any critical changes in conveyance among solid control subjects and TB patients despite the fact that the TGTG+ genotype was higher in control bunch than TB patients (61.25 % versus 57.46% individually). The genotype TGTG- (TGTG erasure) was measurably non critical ($\chi 2$ =0.6637, P=0.4153) in this populace and this genotype was discovered lesser in the two patients and sound control subject gatherings (8.21% and 6.25% respectively).The heterozygous TGTG +/ - genotype dispersion was marginally extraordinary in TB patients and control gathering (34.33% versus 32.5% separately). The TGTG + allele was higher in control gathering (77.5%) than patients (74.63%) while TGTG-allele was lower in charge gathering (22.5 %) contrasted with TB patients (25.37%) however the distinctions are factually non huge ($\chi 2$ =0.6637, P= 0.4153). The carriage rate of TGTG+ allele was practically same in patients and control while the TGTG-allele carriage rate was higher in patients (42.53% versus 38.75%) yet the chances proportion of TGTG-allele is 1.121 and TGTG-allele carriage rate is 0.8919.

Key- words: Pediatric tuberculosis, NRAMP1, SLC11A1 gene, Allele frequency.

Introduction

Tuberculosis (TB) has infected about one-third of the wold's 5 billion people. In 1995, from this pool of infected people, about 3.3 million new active smear – positive cases were reported, along with an estimated 4 million other cases, which led to about 2-3 million deaths. Tuberculosis infection (TB) may result in pulmonary or extrapulmonary complication. One of the more devastating extrapulmonary manifestations is tuberculous spondylitis (STB) [1].At least half of tuberculous musculoskeletal infections eventually present as spondylitis. At our institution STB accounts for 40% of all patients requiring surgery for back problems.

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The association between the incidence of tuberculous spondylitis with the Natural resistance Associated Macrophage Proten1 (NRAMP1, also known as solute Carrier Family 11a member1) polymorphism by studying the genetinc segregation of this polymorphism and the incidence of the disease among members of the West Javanse population undergoing surgery for tuberculous spondylitis at our institution. [3].We compared the distribution of NRAMP1 polymotphism at two specific sites, namely D543N, and 3UTR , 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 spondylites compared to healthy subjects [1-3].

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, these is no association between NRAMP1 polymorphism with the propensity for development of pulmonary tuberculosis or tuberculous spondylitis. In fact, NRAMP1 may provide protection against the development of tuberculous spondylitis. The human homologue of the NRAMP1 gene, designated NRAMP1, has been cloned and mapped to chromosome 2q35.In recent times, number of genes have been investigated in various case control studies, of which the natural resistance–associated macrophage protein (NRAMP1) is thought to be important in the intracellular killing of Mycobacteria [4]. The existence of possible association between NRAMP1 gene polymorphisms and pediatric tuberculosis in Central india. Different clinical characteristics are seen in paedeatric and adult TB patients so gene polymorphisms may have different clinical effects. Although the relationship between malnutrition and TB has been recognised long ago, this has not been studied in Central india. There is also a limited insight into the influence of socio-economic factors on nutritional status of TB patients in Central india. The malnutrition among newly diagnosed paediatric pulmonary TB patients at the time of registration and to assess the impact of socio-economic characteristics of patients on the nutritional status at registration .Tuberculosis has proven to be difficult to wipe out from life, but the sighting of the genetic factors that underlie susceptibility to the disease may in the future be useful to prevent infection and progression to active disease or to determine treatment [3-4].

Tuberculosis (TB), one of the oldest recorded human diseases, is often deadly infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics. TB is primarily a pulmonary disease that is initiated by the deposition of *Mycobacterium tuberculosis*, contained in aerosol droplets, onto lung alveolar surfaces but it has many manifestations, affecting bone, the central nervous system, and many other organ systems. The progression of the disease can have several outcomes, determined largely by the response of the host immune system. Drug-resistant TB is widespread and found in all countries surveyed. It emerges as a result of treatment mismanagement, and is passed from person to person in the same way as drug-sensitive TB. Multidrug-resistant TB (MDR-TB) is caused by bacteria that are resistant to the most effective anti-TB drugs (isoniazid and rifampicin) [6].MDR-TB results from either primary infection or may develop in the course of a patient's treatment. Extensively drug-resistant TB (XDR-TB) is a form of TB caused by bacteria that are resistant to isoniazid and rifampicin (i.e. MDR-TB) as well as any fluoroquinolone and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin).These forms of TB do not respond to the standard six month treatment with first-line anti-TB drugs and can take two years or more to treat with drugs that are less potent, more toxic and much more expensive. While host genetic factors may contribute to the development of MDR-TB, incomplete and inadequate treatment is the most important factor leading to its development, suggesting that it is often a man made tragedy [5-7].

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. 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. Tuberculosis (TB), caused by infection of Mycobacterium tuberculosis, 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. Host genetic susceptibility, together with some environmental and lifestyle factors, has been suggested to contribute to such clinical diversity. The association of several SLC11A1 loci have been extensively investigated, including 39 UTR (1729+55del4), D543N (Asp543Asn), INT4 (469+14G/C), and 59 promoter (GT)n. An ethnicity specific effect of SLC11A1 polymorphisms on TB risk [8-9].

Material and Methods

Study population

The study population included pediatric tuberculosis patients and normal healthy individuals as controls of central Indian population. Informed consent were taken from all the individuals participating in the study. Information related to age, sex, ethnic origin, nutritional status, previous TB history, socio-economic status of the family and associated medical data such as HIV infection are collected from each individual participating the study using a questionnaire.

Patients

There were 134 pulmonary TB patients (age >14)

were included for this study .All patients had established disease. The disease was defined on the basis of clinical manifestations, culture or smear positive, tuberculin test, chest X-ray and bronchoscopy. No patients were HIV infected.

Controls

152 Subjects were included as control for this study. The control group included are pediatric patients not having TB or any other infectious diseases and normal heathy subjects. Control group had no history of TB and had normal radiographic examination findings and tuberculin test result of < 5mm. Control subjects matched with patients by age, sex and ethnicity.

Molecular Analysis:

Sample collection –

Approx. 5ml. of blood samples of each patient and control were collected in storage vials containing EDTA as anticoagulant (50µl. EDTA for 1ml. blood), from the pediatric department of Sanjay Gandhi hospital and Shyam Shah Medical college Rewa, Ranbaxy pathology Regional collection centre Rewa and District hospital Satna, Shahdol and Sidhi .

Isolation of genomic DNA from blood samples:

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.

Qualitative and quantitative analysis of isolated DNA

The isolated DNA have to be used for further molecular analysis DNA preparations were subjected to quatitative and qualitative analysis to test the aptness , the procedures used were described in the following paragraphs.

Quantitative analysis

isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm. And its unit is µg/µl.

Qualitative analysis

Qualitative analysis of genomic DNA isolated from blood samples was carried out by agarose gel electrophoresis. Single band in the gel represents good quality DNA. A horizontal agarose slab gel electrophoresis apparatus (Bangalore Genei, Bangaore, India) was used. In brief, 4-5 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solution (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco*RI / *Hind* III double

digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism screening

Gene polymorphisms were studied using PCR and RFLP. The genomic DNA isolated from peripheral blood of both cases and controls were subjected to PCR followed by restriction digestion and agarose gel electrophoresis. All the PCRs were carried out in a PTC 200 thermal cycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative control was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA which was replaced by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called "star activity" which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO).The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on agarose gel. Gels were stained with ethidium bromide solution (0.5 µg/ml) and subsequently visualized and photographed under UV transilluminator.

NRAMP1 / SLC11A1 Genotyping by PCR-RFLP

In the present study polymorphisms in 3'UTR region of NRAMP1 gene was analized. The 3'UTR polymorphism is a TGTG deletion in the 3' un-translated region.

Polymerase chain reaction

PCR Mix

The PCR was carried out in a final volume of 25 µl, containing 100 ng of genomic DNA(4-5 µl), 2.5 µl 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 concent ration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of secific forward and reverse primers and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalore genei).

PCR Thermal cycle profile

Initial denaturation was allowed for 3 minutes at 94^0C , then 35 cycles of denaturation at 94^0C for 1 min, annealing at 55^0 C for 1min,extention at 72^0C for 1min and final extention for 3 min at 72^0C in a thermal cycler. The 3' UTR region was amplified using forward primer 5′-GCATCTCCCCAATTCATGG -3′ and reverse primer 5′-AACTGTCCCACTCTATCCTG -3′. A ladder with amplified product has been run on 1% agarose gel. The amplicon had a size of 244 bp.

Restriction fragment length polymorphism

The PCR products were digested with the restriction enzyme Fok I (New England Biolabs,USA) by incubating at 37^0 C for overnight, and the resultant products were analyzed on 2% agarose gel stained with ethedium bromide for RFLP analysis of 3'UTR gene . Recognition site for FokI is

5 'GGATG$(N)_9$▼…….3'

3'CCTAC$(N)_{13}$▲…….5'

Genotyping

FokI cuts the gene homozygous for $TGTG^+$ allele into two bands of 211bp and 33 bp, these individuals were scored as TGTG +/+ , and the gene homozygous for TGTG deletion produces a 240 bp band which was scored as TGTG +/ delete. Individuals heterozygous for TGTG deletion produces bands of 211bp, 33 bp and 240 bp, these were scored as TGTG +/delete.

Statistical analysis

To check the difference in the anthropometric and biochemical parameters of tuberculosis patients and the non-tuberculosis controls we used student's t test. The difference in the biochemical and anthropometric parameters between diabetic and healthy individuals in the controls was also assessed using the nonparametric student's t test. The P-values calculated using t test along with the mean (inter-quartile range) were presented. Statistical analyses were performed using statistical package, Prism 3.0, Prism 5.1 version.

Results and Discussion

Detection of Genenic Polymorphism in NRAMP1 Gene:

FokI cuts the gene homozygous for $TGTG^{+}$ allele (TGTG +/+) into two bands of 211bp and 33 bp, , and the gene homozygous for TGTG deletion (TGTG -/-) produces a 240 bp. Individuals heterozygous for TGTG deletion (TGTG +/delete) produces bands of 211bp, 33 bp and 240 bp.

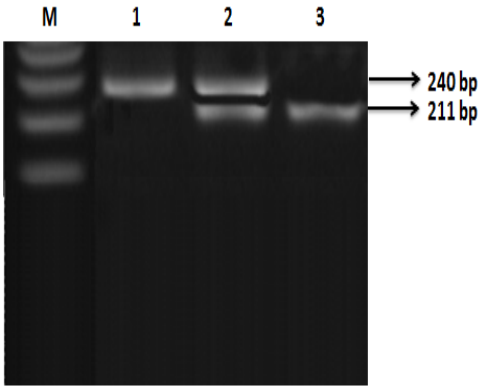


Fig. 1: Representative gel picture of NRAMP1 gene

M= 100 bp molecular marker, Lane 1=homozygous mutant genotype, Lane 2=heterozygous gene, Lane 3= wild type genotype. The 33-bp fragments are not visualized.

The distribution of NRAMP1 3'UTR polymorphism (rs 17235416) was under Hardy Weinberg equilibrium (HWE) in TB patients as well as in control subjects. The observed genotype frequencies, allele frequencies and carriage rates for NRAMP1 3'UTR polymorphism are illustrated in table 2 , 3 and graph 3.2.1, 3.2.2, 3.2.3. TGTG +/+ genotype did not show any significant changes in distribution among healthy control subjects and TB patients even though the TGTG+ genotype was higher in control group than TB patients (61.25 % vs. 57.46% respectively). The genotype TGTG- (TGTG deletion) was statistically non significant (χ^2 =0.6637, P=0.4153) in this population and this genotype was found lesser in both patients and healthy control subject groups (8.21% and 6.25% respectively).The heterozygous TGTG +/- genotype distribution was slightly different in TB patients and control group (34.33% vs 32.5% respectively), and had an odds ratio of 1.086 that suggests that this genotype had no association with pediatric TB in this population. Even though the overall genotype was statistically non significant the TGTG+/+ genotype was higher in healthy control group so it may be protective in this population.

The TGTG + allele was higher in control group (77.5%) than patients (74.63%) while TGTG- allele was lower in control group (22.5 %) compared to TB patients (25.37%) but the differences are statistically non significant (χ^2 =0.6637, P= 0.4153). The carriage rate of TGTG+ allele was almost same in patients and control while the TGTG- allele carriage rate was higher in patients (42.53% vs 38.75%) but the odds ratio of TGTG- allele is 1.121 and TGTG- allele carriage rate is 0.8919, this indicates the carriage rate of this alleles are not associated with pediatric pulmonary TB in this population. The data obtained for genotype distribution, allele frequency and carriage rate suggests NRAMP1 3'UTR polymorphism is not statistically associated with pediatric TB in Central Indian population.

Table 1: Frequency distribution and association of Genotype, allele frequency and carriage rate of NRAMP1 3'UTR polymorphism in central Indian population using Chi Square Test

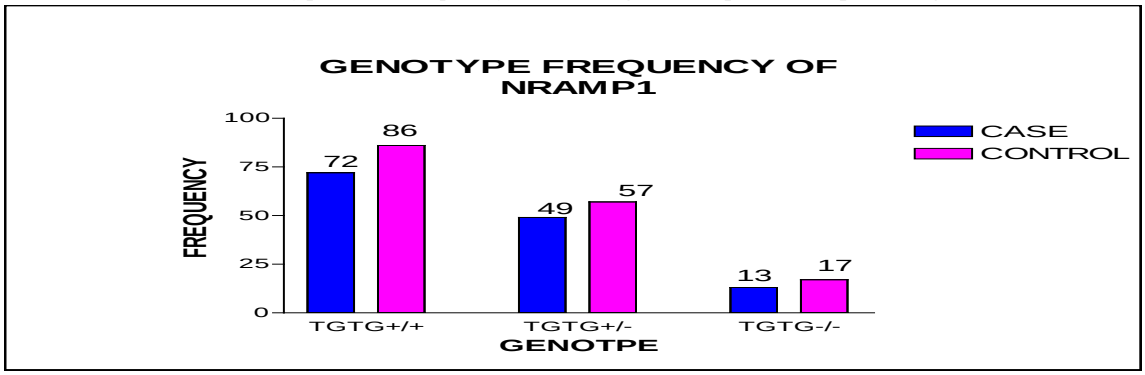
NRAMP1 Gene	CASE N=134 N (%)	CONTROL N=160 N (%)	CHI SQARE VALUE $\chi 2$ (P Value)
Genotype			
TGTG+/+	77 (57.46)	98 (61.25)	0.6407 (0.7259)
TGTG+/-	46 (34.33)	52 (32.5)	
TGTG-/-	11 (8.21)	10 (6.25)	
Allele			
TGTG+	200 (74.63)	248 (77.5)	0.6637 (0.4153)
TGTG-	68 (25.37)	72 (22.5)	
Carriage Rate			
TGTG+	123 (91.79)	150 (93.75)	0.2700 (0.6034)
TGTG-	57 (42.53)	62 (38.75)	

N – Number of individuals in study group; %- Genotype allele frequency and carriage rate expressed in percentage

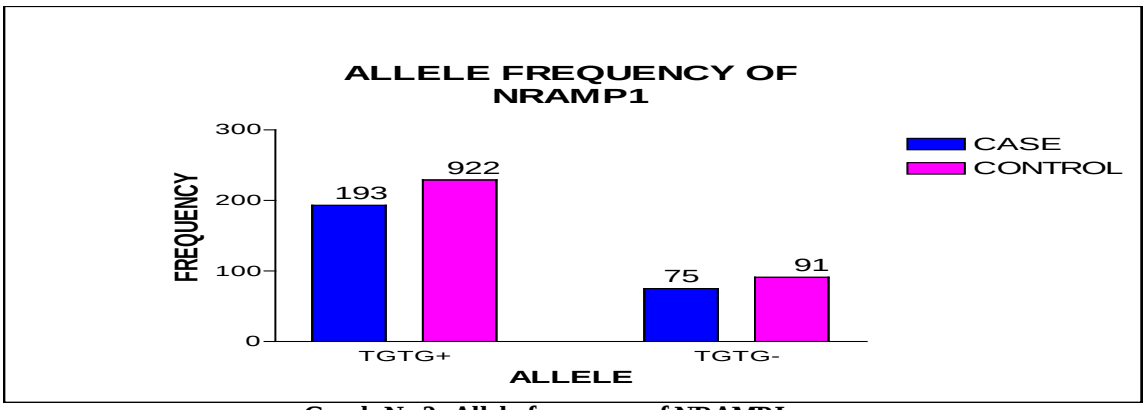
Table 2: Fisher Exact Test values of NRAMP1 polymorphism

NRAMP1 Gene	CASE N=134 N (%)	CONTROL N=160 N (%)	P Value	Odds Ratio (95% CI)
Genotype				
TGTG+/+	77 (57.46)	98 (61.25)	0.5516	0.8546 (0.5355-1.364)
TGTG+/-	46 (34.33)	52 (32.5)	0.8040	1.086 (0.667-1.766)
TGTG-/-	11 (8.21)	10 (6.25)	0.6503	1.341 (0.5513-3.264)
Allele				
TGTG+	200 (74.63)	248 (77.5)	0.4376	0.8539 (0.5838-1.249)
TGTG-	68 (25.37)	72 (22.5)		1.171 (0.8007-1.713)
Carriage Rate				
TGTG+	123 (91.79)	150 (93.75)	0.6595	0.8919 (0.5793-1.373)
TGTG-	57 (42.53)	62 (38.75)		1.121 (0.7282-1.726)

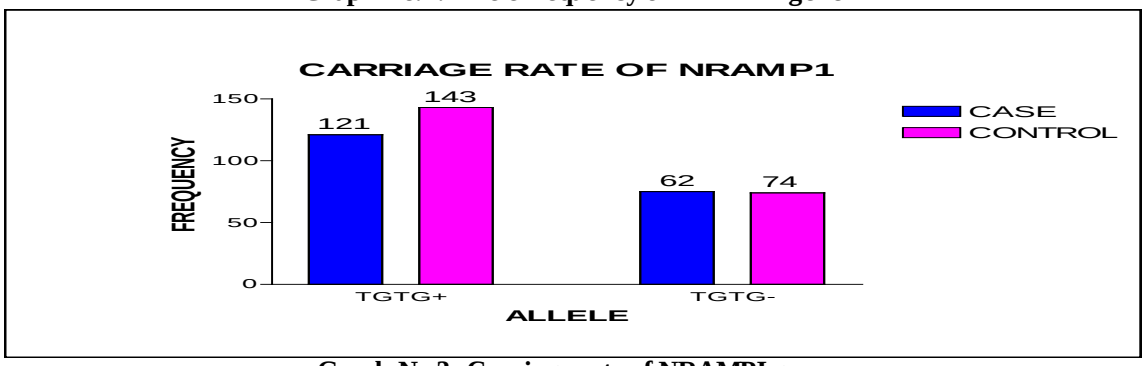
N – Number of individuals in study group
%- Genotype allele frequency and carriage rate expressed in percentage



Graph No.1: Genotype frequency of NRAMPI gene



Graph No.2: Allele frequency of NRAMPI gene



Graph No.3: Carriage rate of NRAMPI gene

Association of Nutritional Status with TB:

Nutritional status was analyzed using the weight for age method and BMI. Nutritional status and BMI was identified as a significant risk factor for TB (P= <0.001) in children. Almost 50% 0f the patients are severely malnourished and only 9.7% patients are normal. The observed data in the study are shown in table.

Association of Socio-economic Factors with Pediatric Tuberculosis

Age and sex did not show any association with TB. Among the socio-economic factors analyzed mothers' occupation and fathers' education and occupation are not associated with TB in children while, mothers' education showed significant association with TB (P= 0.0162) in children. House condition (P=0.0041) and person per bedroom (crowded environment) (P= 0.0027) and income per person (0.0111) also showed significant association with TB.

Table 3: Association of nutritional status with pediatric tuberculosis

Characteristics	Case N=134	Controls N=200	P value
Age (years, mean +/- SD)	6 +/- 0.8	6 +/- 1.2	1
Weight	10 +/- 5.89	14 +/- 6.8	<0.0001***
BMI	12.5 +/- 1.4	19 +/- 2.1	<0.0001***
Nutritional Status			
Severe malnutrition (%)	66	20	<0.0001***
Moderate malnutrition (%)	52	28	
Normal (%)	13	112	

Table 4: Association of socio-economic factors with pediatric tuberculosis

Characteristics	Case N=134	Controls N=200	P value
Age			
<5	71	86	0.8958
5<10	63	74	
Sex			
Male	60	73	0.8842
Female	74	87	
Mothers'educationed			
No education	75	56	0.0162
Primary education	40	48	
Secondary education	15	27	
Higher secondary education	12	21	
Graduation and above	2	8	
Mothers' occupation			
House wife	38	51	0.0942
Daily labour	64	65	
Business	18	29	
Fathers' education			
No education	56	61	0.3210
Primary education	43	42	
Secondary education	19	28	
Higher secondary education	14	21	
Graduation and above	2	28	
Fathers' occupation			
Daily labour	83	90	0.3697
Business	45	57	
Service	6	13	
Daily labour	83	90	0.3697
Business	45	57	
Service	6	13	
Persons per bedroom			
More than two persons	33	66	0.0027 **
Two persons or less	101	94	
House conditions			
Poor	42	31	0.0041**
Fair	81	106	
Good	11	23	

Globally, tuberculosis (TB) continues to be an unacceptably high toll of disease and death among children, particularly in the wake of the HIV epidemic. Tuberculosis holds higher risk of severe disease and death among young children than adults (WHO, 2007). Studies in the animal and human host have constantly demonstrated reduced microbial killing and diminished monocyte recruitment to the site of infection in infants than adults. Scientific and clinical research efforts have focused on TB in adults on the other hand, children are particularly vulnerable to severe disease and death following infection, and those with latent infection become the reservoir of disease reactivation in adulthood, fueling the upcoming epidemic. [12].So studies are needed in childhood TB, which would provide wider insights

and opportunities to facilitate efforts to control this ancient disease. As it is a multifactorial disease, it is difficult to distinguish between exposure to the bacteria and infection with the bacteria. But it is clear that only a small portion of the infected develop clinical TB. [13].Disease development depends on the host and environmental factors. Environmental factors may include the bacterial virulence factors and the mode of exposure, the host factors may be genetic such as mutation in genes associated with immunity and nongenetic such as age, smoking or alcoholism, medical conditions and socio-economic status. A household source is most commonly fretful for young children but older children are increasingly likely to be infected outside the household. Poverty, poor housing, urban environments and overcrowding are all associated with increased transmission [10-14].

Malnutrition is also having an important role in TB susceptibility. Many observational and experimental animal data and impaired CMI observed in malnutrition, support its role as a risk factor for childhood TB. The outcome of exposure and infection may be based on a combination of both environmental and host factors. Pediatric TB is clinically different from adult TB and findings on the association of the effects of gene variants on pediatric TB will provide further information about the molecular & genetic mechanisms involved in the pediatric TB development and also help in the development of more well-organized methods to prevent TB control. It is mostly applicable for decreasing adult TB developed from latent childhood TB infection. [13]. Many candidate genes have been studied in different populations to understand the association with TB but the results were inconsistent and most of the studies were conducted in adults. In present study we analyzed the association of NRAMP1, gene polymorphisms with susceptibility to pediatric TB and the association of socioeconomic factors with pediatric TB. NRAMP1 gene appears to play a role in the pathophysiology of a number of intracellular infections, including mycobacteria (Bellamy *et al*., 1998; Blackwell *et al*., 2001). To find out the association between NRAMP1 (SLC11A1) gene polymorphism and tuberculosis many studies have been conducted in different populations. The results obtained were not consistent and reported that the susceptibility of polymorphic variants to tuberculosis is population specific. Delgado et al. (2002) reported that the frequencies of genetic variants of NRAMP1 gene are population-specific and discrete environmental and natural selective factors have probably resulted in population-specific immune-genetic adaptations to clinical TB. [12,14]. Later Li et al. (2006) reported that studies suggested an ethnicity specific effect of NRAMP1 polymorphisms on TB risk. The study also reported the four gene polymorphisms that have been extensively studied for NRAMP1 [3′-UTR, D543N, INT4 and 5′-(GT)n] do not show a significant association with tuberculosis in a European population but significant association among most of these variants has been found in African and some Asian population. This underline the need for understanding the frequency of a particular polymorphism in specific populations before assigning to it specific infectious-disease associations. [16].Polymorphism in the 3'UTR region have a strong association with tuberculosis susceptibility and subjects heterozygous for this variants were four times higher among patients with tuberculosis than those bearing the most common NRAMP1 genotype in West African population. Richard et al. (1998) reported subjects heterozygote for 3'UTR (TGTG) variants are at increased risk for TB in Gambia [8-17].

A study from Guinea-Conakry reported that 3'UTR polymorphisms are significantly associated with tuberculosis. A study conducted among a high-incidence community in South Africa reported that homozygotes for the TGTG deletion (1729+55del4) in the 3'UTR of NRAMP1 showed susceptibility to TB. Study from Poland reported that 3'UTR polymorphism is associated with susceptibility to infection. 3'UTR polymorphisms were associated with pulmonary TB (Liu and TB in Chinese Han population [15-20].

Later another study reported that polymorphism of 3'UTR locus in NRAMP1 gene might affect their susceptibility to TB in Han population living in the northern part of China. Jin et al. (2009) reported TGTG+/delete and TGTG delete/delete genotypes at the 3'UTR locus in the SLC11A1 (NRAMP1) gene were associated with pediatric tuberculosis in China. The 3'-UTR del/del genotype appeared to be associated with susceptibility to PTB in patients aged less than 30 years in contrast, TGTG+/del might be associated with resistance against the development of active PTB in Tunisian population.In India, a study conducted in south India suggests that NRAMP1 gene polymorphisms [(CA)n, 823 C/T, TGTG+/del and D543N G/A] may not be associated with the susceptibility to pulmonary and spinal TB in the Indian population[18,20]. Singh et al. (2011) reported

that a significant association between NRAMP1 gene variants, 577G/A and INT4, with PTB susceptibility and subsequent disease progression in East India.But 3'UTR (TGTG) variants did not show any association with pulmonary TB in Morocco, African Americans and Caucasians, Mexican mestizo population, Iranian and Indonesia [17-22].

In our study the data obtained for genotype distribution, allele frequency and carriage rate did not show significant association with pediatric pulmonary TB in Central Indian population. The TGTG + genotype was higher in control group than patients (OR=0.8546) so it may have slight protective role on pediatric TB in this population. The heterozygous TGTG +/- genotype had an odds ratio of 1.086 that suggests that this genotype had no association with pediatric TB in vindhyan region population. The mutant TGTG- genotype was low in patients as well as control group. The TGTG +, TGTG- alleles (OR=0.8539 and OR=1.171 respectively) and the carriage rate (OR=0.8919 and OR=1.121 respectively) of these alleles are not statistically associated with pediatric pulmonary TB in this population. Our result was supported by the study from south India, reported by Selvaraj et al in 2002, that NRAMP1 gene polymorphisms may not be associated with TB in population. The study reported by Baghdadi et al, Velez et al, Nino-Morino et al, Muayard Merza et al ,Sahiratmaja et al and Hatta et al supports our study. A study from China reported that TGTG-/- genotypes in the NRAMP1 gene were associated with pediatric TB (Jing Jin *et al*., 2009) .The difference in results obtained from different populations can be explained by the report of Delgado et al that the frequencies of genetic variants of NRAMP1 gene are population-specific and discrete environmental and natural selective factors have probably resulted in population-specific immunogenetic adaptations to clinical TB. Genetic variants of NRAMP1 polymorphisms may serve as markers of unidentified genetic factors that may play a significant role in host immunity to TB in different populations. But it is not clear that other functional polymorphisms exists in NRAMP1 gene or other genes so to elucidate the issue further studies are required on the function of this gene and its genetic variants [25-17].

Socio-economic indicators of a population are overcrowding, poverty, income, public assistance, employment and education. All this factors are associated with TB, TB risk is increased with low socio-economic indicators. Wilson stated that Tb mortality was declined in US, before the introduction of isoniazid and effective chemotherapy, by improving housing, sanitation and general socio-economic status. [19]. Reichman and O'Day concluded in their study that socio-economic status was correlated with TB infection. The socio-economic status of the healthy controls was found to be comparatively higher than the TB patients at different stages of treatment in studies conducted (Tungdim *et al*., 2008). In the present study we analyzed the association of pediatric pulmonary tuberculosis with different socio-economic factors such as education, housing condition, employment, crowding and income. The data obtained from the study suggested that mothers' education is inversely associated with TB in children (P=0.0162). This may be due to the poor knowledge of TB or infection because of poor education.Same result was reported by Mohamed et al. It supports our result that suggests that mothers' education is associated with TB risk in children. But in this study fathers' education didn't show any involvement with TB risk in children [18-21].

The difference in the association of parents' education may be due to the prime role of mothers on caring children or educated mother supposed to have better knowledge about health. Data obtained from our study showed the association of fathers' and mothers' occupational status with pediatric TB was not statistically significant (P=O.3697 and P=0.0162). Housing condition showed significant association with TB in children (P=.0.0041). The possible explanation of this result may be the known reasons that a viable bacillus exposed to sunlight (house with enough ventilation) is often phenotipically weak to initiate an infection and it prologs contact with infectious droplet nuclei. A poor housing condition with high dampness enhances the viability of the bacillus that increases the chance of transmission and development of TB. [26,28,30].Same result was found in a study conducted among adult Indians by Dheeraj Gupta supports our result. While a study in rural area of Bangladesh reported that the childhood TB is not associated with housing condition. The difference in the results may be due to the dissimilarities in the basic hosing conditions and house hold items in two countries.Number of persons per bedroom, which indicates crowding, showed significant association with TB in children (P=0.0027) because more persons occupies within an area decreases the sharing space results in increased exposure to *M tuberculosis*. Mangtani et al and Druncker et al reported overcrowding increases the risk of disease

transmission. Mohamed et al also reported that crowding increases the risk of TB in children. These reports support the present study result.Income per person also showed a significant association with pediatric TB (P=0.0111) in our study. The basis of it may be that the insufficient income is responsible for poverty which in turn results in poor nutrition. Poor nutrition renders immune system easily vulnerable to invading organisms. Singh et al reported that childhood TB in the community are inversely vary with their economic level. Same result was also reported by Gupta et al. These earlier reports support our result.Severe malnutrition is probably associated with TB than other chronic diseases [25,28].Cegielsky and McMurray also found that malnutrition appeared to increase the risk of developing tuberculosis. A study conducted among the adult tribal population of Northeast India by Mary Grace Tungdim and Kapoor reported that malnutrition is significantly associated with TB.In contrary reports suggests TB itself causes wasting. Tungdim and Kapoor reported that there was depletion of fat stores and muscle wastage with TB but with TB treatment there was improvement in fatness level, muscle strength and mass. Onwubalili described that TB patients had significant reduction in the body mass index, triceps skinfold thickness and arm muscle circumference [29, 30].Cegielski et al reported that impaired CMI observed in malnutrition from experimental studies support its role as a risk factor for childhood TB. So from observational and experimental data we can assess malnutrition is a risk factor for TB.In our study malnutrition was assessed by body mass index (BMI). The data showed that BMI was poor in TB patients than healthy controls and the study also showed a significant association between malnutrition and pediatric pulmonary TB (P=0.0001). This result was supported by the reports of Tverdal, an increasing risk of pulmonary TB with decreasing body mass index for both sexes and all age groups, Cegielsky and McMurray, malnutrition increases the risk of developing tuberculosis and Mary Grace Tungdim and Kapoor [22-31].

Conclusion

In summary, we examined the association of NRAMP1, gene polymorphisms with pediatric pulmonary TB. We also studied the association of socio-economic factors and malnutrition with pediatric pulmonary TB. The study included 134 pulmonary TB patients and 160 healthy controls for polymorphic studies and to study the association of socio-economic factors and malnutrition with pediatric pulmonary TB. In polymorphic studies the results obtained are.The Nramp1 3'UTR (TGTG deletion) polymorphism didn't show statistically significant association with pediatric pulmonary TB in central Indian population. The statistical values of TGTG +/+ genotype was higher in control group than patients, suggests that this genotype may have protective role in pediatric pulmonary TB in this population..In socio-economic factors mothers' education, housing condition, persons per bedroom and income per person are significantly associated with pediatric TB but fathers' education and occupation, mothers' occupation did not show significant association with pediatric TB. The nutritional status was examined by weight for age method and BMI. The nutritional status is observed as a risk factor for pulmonary TB in children.In conclusions, our study suggests that polymorphism in Nramp1 (TGTG deletion) is likely to be a risk factor for pediatric pulmonary tuberculosis in central Indian population . The study also suggests that nutritional status and socio-economic factors like mothers' education, housing condition, persons per room and income per person are risk factors for pulmonary TB in children. To draw a conclusion about the association of gene polymorphisms with susceptibility to tuberculosis (TB) in humans is difficult because conflicting results were obtained from different studies performed in different populations. The inconsistency between the studies may be due to various factors including ethnicity-related differences in gene polymorphisms, differences in clinical severity of the patients between studies, unknown cofactors like socio-economical factors, nutritional status, other co-infections or different genetic interactions or whether another functional polymorphism exists within the gene. The study of ethnic-specific genetic associations with TB susceptibility may lead to a novel method of TB therapy and prophylaxis in an ethnic-specific manner. Importance of socio-economic factors in TB risk has been frequently reported by several studies conducted among different populations in the world. The existing treatment-based approach to tuberculosis control cannot be expected to lead to eradication of tuberculosis so it is important to initiate efforts in prevention through improvement in socio-economic and nutritional status. Research priorities and advances in pediatric TB research may also provide wider insights and opportunities for TB control. Regional data on the epidemiology of childhood TB would in turn help to understand about current transmission and the effectiveness of control strategies. The molecular studies would contribute to

better understanding of the pathogenic processes that underlie major infectious diseases and offer hopeful new antimicrobial agents or immunomodulation therapies by allowing a more systematic study of the genetic influences. However, as the present study was performed with relatively small sample size, further studies with larger sample sizes would be necessary to elucidate the role of host gene polymorphisms in tuberculosis.

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