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HOST GENE POLYMORPHISM INCREASING SUSCEPTIBILITY TO TUBERCULOSIS AMONG PATIENTS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT
Back ground: Tuberculosis (TB) is a major health problem globally. Immune dysfunction is found to be associated with the increased incidence of TB. Immune dysfunction in diabetes mellitus (DM) increases the risk of developing active TB. Host genetic factors were described to be one of the possible risk factors for TB susceptibility. This study aimed to look for FOKI polymorphism of Vitamin D receptor and the polymorphism of Solute Carrier Family 11 member 1 (SLC11A1) genes 3'UTR, D543N and INT4.
Method: This was a prospective case-control study. Patients with type 2 DM and TB were recruited as cases and patients with type 2 DM without TB as controls. The host gene polymorphism was determined using PCR-RFLP.
Result: Among the SLC11A1 genes tested, the TG/TG +/- heterozygous allele of 3'UTR and the G/G homozygous wild type allele of D543N varied significantly between the patients with DM andTB when compared with the patients with control arm. FOKI polymorphism of the vitamin D receptor and the INT4 polymorphism of SLC11A1 did not vary between cases and controls.
Conclusion: The allelic forms of 3'UTR and D543N of SLC11A1 genes were found to be significantly associated with TB among patients with type 2 DM.

KEYWORDS: Tuberculosis, Diabetes, SLC11A1, FOK1

1.INTRODUCTION

Tuberculosis (TB) is one of the top 10 causes of death and the leading cause from a single infectious agent worldwide. Global TB report of WHO estimates that there were ten million incident cases of TB globally in 2021[1]. Active TB develops in 10% of the exposed individuals and in the remaining 90%, there is containment of infection which is termed as latent tuberculosis infection **(LTBI**).[2] Immune dysfunction in individuals such as diabetes can lead to reactivation of LTBI to active TB.[3] But all those with immune dysfunction do not develop active TB. Host genetic factors were described to be one of the possible risk factors for TB susceptibility.[4] This study is aimed to determine the prevalence of FOK1 gene polymorphism of Vitamin D Receptor (VDR) and polymorphism of 3'UTR, D543N and INT4 of SLC11A1 genes among patients with type 2 DM and to estimate its association with TB.

2.MATERIALS AND METHODS

This study was a prospective case control study and was approved by the institutional review board. The inclusion criteria for the study subjects include patients with type 2 DM pat the age of >25 years and on DM treatment with oral hypoglycemic agents (OHA) / insulin for a minimum period of three months. Patients who were HIV positive and those with any immunosuppressive therapy were excluded from the study. Blood samples were collected from the patients with the informed consent. Patients with type 2 DM and TB (no=38) were recruited as cases and type 2 DM patients without TB (no=38) served as control arm.
All clinical specimens were processed in TB containment laboratory in the Biosafety level III laboratory. Mycobacterial laboratory diagnosis of TB was based on *M.tuberculosis* positive on culture by MGIT Mycobacteria Growth Indicator Tube and Xpert MTB/RIF assay.

2.1 DNA extraction

DNA was extracted by Gentra Kit Manual method (Qiagen, Hilden, Germany)[5] and quantified using Thermoscientific Multiskan Go; Type 1510 Spectrophotometer; (Thermo Fisher Scientific Oy; Finland). The primers and the PCR cycling conditions were followed as previously described.[6]

2.2 FOK I polymorphism

FOK I polymorphism was tested in the study subjected as described by Errouagui et al.[7] The presence of a FOKI site indicates the presence of the first ATG and is designated by f whereas the absence of the FOKI site is designated by F.[8] The wild type allele FF which was not digested by the enzyme has the band at 265bp. The homozygously mutant allele ff has the digested product at 196bp and 69bp. For the heterozygously mutant allele Ff bands were observed at 265bp, 196bp and 69bp.

2.3 The SLC11A1 genotyping

Polymorphism of INT 4, D543N and 3'UTR genes of the SLC11A1 genes were tested in the study subjects as described by Stagas MK et al.[9] INT4wild type does not have the site for the restriction enzyme and has the band at 623bp and the G→C mutant type show two bands of 455bp and 169bp. The enzyme ApaI was used for INT-4. A non-conservative single base substitution at codon 543 that changes aspartic acid (Asp) to asparagine (Asn) was termed D543N. Enzyme AvaII was used for D543N with allele G (Asp) showing three bands of 126bp, 79bp and 39bp, and A (Asn) showing two bands of 201bp and 33bp. A TGTG deletion in the 3' un-translated region was termed 3′UTR. The 3'UTR starts from 55 nt 3′ to the last codon in the exon 15. In this region TGTG deletion was found. FOKI was used for 3UTR with allele TGTG+ showing 211bp and 33bp fragments and del showing a 240bp

2.4 Statistical Methods

Data was entered in Microsoft Excel (2013) spreadsheet and the analysis was performed using IBM SPSS (Statistical Package for the Social Sciences) version 21. The association between two categorical variables was analyzed using chi-square test. The p value for the statistical significance was set at <0.05 in this study.

3. RESULTS

Among the 38 patients with type 2 DM and TB, 27 (71%) patients had pulmonary TB (PTB) and 11(29%) had extra pulmonary TB (EPTB) of which 29 (76%) were male and 9 (24%) were females. The mean age was 55 years (36 - 75 years). Among the cases 17 were from North India and 21 were from south India. Smear positivity was seen in 27 patients and smear negative in 11 patients. Rifampicin resistance was seen in 5 of the 38 patients. While in the control group of patients 17 (45%) were male and 21 (55%) were females and the mean age was 57 years (38-76 years). Among the control group of patients 5 were from north India and 33 were from south India.

The allelic frequencies of the genotypes, FO1, INT4, D543N and 3'UTR among cases and controls is presented in table-1. Individuals who have wild type alleles of D543N are more likely to get TB as compared to patients who had mutant type which is statistically significant (p=0.002). In respect to 3'UTR, the individuals who have mutant alleles of 3'UTR are more likely to get TB as compared to patients who had wild type which is statistically significant (p=0.028). The polymorphism of FOK1 and INT4 did not show statistical significance between the cases and the controls.

The distribution of the allelic forms of FOK1, INT4, D543N and 3'UTR between males and females in both cases and controls is listed in table-2. With the 4 genes analysed, males had an increased proportion of wild type allele compared with that of the females in the study. The distribution of the alleles FF, Ff and ff among men were 52%, 44% and 4% and among women were 43%, 43% and 13% respectively. Wild type of D543N and 3'UTR is seen more among men and the heterozygotic D543N and 3'UTR is more among women. With INT4 allelic forms there is no gender variation seen.

The numbers of PTB cases were more among the individuals having the mutant allele of 3' UTR, INT4 and FOK1 when compared with the wild type alleles as presented n figures1, 2 &3. However the smear positivity is more among the individuals with wild type allele compared to that of the mutant alleles. There was equal distribution of wild type and the mutant alleles of D543N among the patients with PTB and EPTB as presented in figure 1-d. Susceptibility to rifampicin was found more among the wild type of FOKI and the mutant type of INT4. The rifampicin susceptibility did not differ between the mutant and the wild type alleles INT 4 and D543N. Among the individual genes tested, the percentage of wild type allele predominated in INT4 gene while the mutant allelic forms predominated in FOK1, D43N and 3'UTR.

4. DISCUSSION

Immune response to MTB infection and progression to clinical disease vary with individuals as only 10% of the infected people infected with MTB develop active TB disease. The important causes which influence the development of active TB include HIV infection, Diabetes mellitus (DM), immunosuppressive drugs and malnutrition. [2]About 50% of the patients with TB are hyperglycemic.[10] Researches suggest the role of host genetic factors in the individual's susceptibility to MTB infection and development of active TB disease. In this study we have assessed the association of genetic polymorphism of 4 genes in type 2 DM patients with and without TB. Supplementation of Vitamin D in individuals with active TB has been found to have better outcome. Calcitrol, the active form vitamin D increases the individual's immunity to tuberculosis mediated by vitamin D receptor (VDR).[11] The VDR gene is located in the chromosomal 12q13 region. There are about 63 polymorphism reported for VDR

genes. The most common polymorphisms of the VDR gene are the BsmI, FOKI, TaqI and ApaI.[12] FOKI is located in exon 2 of the VDR gene and the presence of this polymorphism results in a shortened VDR protein due to an alteration in the start codon. Individuals who have an ACG codon instead of the first ATG codon initiate from the second ATG and have a VDR that is three amino acids shorter. The polymorphisms of FOKI can alter the amount of VDR produced. The FOKI restriction site is a functional polymorphism site of the VDR gene and is related to plasma vitamin D levels in TB patients. [13]To date, the polymorphisms of FOKI have been studied in relation to the risk of TB in many populations; however, the results remain contradictory. [14]

The frequency of FOK1gene which codes for vitamin D receptor among the patients with type 2 DM in our study (irrespective of the cases and controls) were FF:49%, Ff:43% and ff:8%. The frequency of the heterozygous Ff allele is found to be more common in patients with type 2 DM polish population.[15] but the study conducted among the patients from south India shows wild type (FF) is increased.[16] This could be because of ethnic variation in VDR gene polymorphisms. The frequency of the different polymorphic forms of FOK1gene; FF, Ff and ff seen with other studies in patients with TB is comparable with our studies. There was no variation with the frequency of different allelic forms of FOK1 between cases and controls. This is in accordance with the observation of Shang.CA et.al who also did not find association of FOKI polymorphism with TB risk.[17] Vitamin D deficiency has been established to be associated with increased susceptibility to TB. FOK1 codes for vitamin D receptor which is the site of attachment for the biologically active form of vitamin D;calcitriol. Calcitrol increases the defense capacity of macrophages inducing their differentiation, phagocytic capacity, and antimicrobial activity.[18] With our study there is no variation in disease activity with FOKI polymorphism as presented in figure-1-a. The fact that patients with type 2 DM have vitamin D deficiency[19] and exposures to sunlight and dietary factors can influence serum vitamin D concentrations are the plausible explanation for mechanisms other than FOKI polymorphism for TB susceptibility in patients with type 2 DM. The ff homozygote however was reported to have a pronounced risk to TB among Asians.[20] The frequency of ff allele in our study population was less (3 out of 38 in each group) and so the association of ff polymorphism with TB could not be interpreted.

NRAMP1 (Natural resistance associated macrophage protein) is found integral in the membranes of macrophage and neutrophil phagosomes.[21] This protein functions as a divalent metal-proton transporter. NRAMP1 pumps out the Fe2++ ion from the phagosome, and thereby deplete the phagosome of iron. This leads to a reduced availability of iron for intracellular bacteria along with an activation of anti-microbial immune effector mechanisms due to counter-balancing the negative regulatory effects of iron on IFN-γ inducible immune pathways.[22] Iron is important for the growth and multiplication of MTB. Depletion of iron in the phagosome inhibits the propagation of MTB. Mutation in the SLC11A1 genes result in reduced pumping out of iron from the phagosome. Thus with the increased iron content inside the phagosome, MTB multiplies predisposing the patient to development of active TB disease.[23]

The SLC11A1 alleles were seen in many genes. Of those genes, 3'UTR, INT 4 and D543N are the important ones which are found to be associated with the risk for TB. The possible association of SLC11A1 polymorphisms with susceptibility to development of TB has been studied in different countries. However the reports are controversial.[24] The single nucleotide change at codon 543 changing aspartic acid to asparagine is the D543N polymorphism of SLC11A1. In our study, heterozygous G/A allele is the predominant allelic form of D543N in patients with type 2 DM. The G/G homozygous wild type is reported to be predominant in a study from Greece[9] and the frequency of heteromutant G/A was found to be predominant from Indonesia among the patients with TB thus showing the ethnic variation in the polymorphism. [25] In both the studies the A/A homozygous mutant were not seen. However one of the cases in our study population had A/A homozygous mutation. With our results, the heterozygous mutant allele G/A of D543N is higher among cases compared with that of the controls. The frequency of wild type G/G allele was less among the control group than among the cases.

There was a statistically significant variation between cases and controls with polymorphism of 3'UTR. The frequency of the mutant (homozygous or heterozygous) allele is higher compared to the wild homozygous allele. The meta analysis done by Xiang Lei Wi et al also showed an increased susceptibility to TB with the mutant allelic forms TG/del +/- and del/del-/- compared with TG/TG +/+Wild homozygous. Homomutant allele of 3'UTR region was in higher number in our study. The association of TB with 3'UTR polymorphism was not found to be statistically significant among the European population.[29]

With the INT 4 gene polymorphism, there was no statistically significant variation between the cases and controls. Frequency of the INT 4 wild type allele is increased both in cases and controls. There was no homozygous mutant allele of INT4 and the absence of homozygous mutation in INT4 was reported by other studies also.[30] The heterozygote form of INT4 is found to be present only among the PTB and none among the EPTB cases as presented in Figure-1b. The heterozygote allele of D543N and 3'UTR and the wild type of INT4 predominate in the patients with type 2 DM of our study. With gender variation, the wild type of D543N and 3'UTR is seen more among men and the heterozygotic D543N and 3'UTR is more among women. With other allelic forms there is no gender variation seen. An increased association was found with the polymorphism of 3'UTR and D543N genes of SLC11A1 with the type 2 DM patients with TB in our study population.

5. CONCLUSION

To summarize, among patients with type 2 DM, FOK I polymorphism of the vitamin D receptor did not vary between the individuals with and without TB. There is increased frequency of FF allele in our study group (both cases and controls) Among the 3 genes of SLC11A1 studied, polymorphism of D543N and 3'UTR varied significantly between the cases and the controls. The pattern of allelic frequency varied between the genders. The wild type alleles were more predominant in males and the heteromutant alleles predominated in women.

The relatively less number of study subjects and not testing the gene polymorphism among patients without TB and DM are the two important limitations of our study. Studies including more number of cases and controls are needed for further confirmation of our observation.

6. Conflict of interest: None

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Table Captions
Table 1: Logistic regression analysis of gene polymorphism between cases and controls
Table 2: Gender distribution of gene polymorphism in the study subjects

Figure Captions
Figure 1 (a-d): Disease activity with different allelic forms among Type 2 DM patients with TB

Table 1: Logistic regression analysis of gene polymorphism between cases and controls

Gene	Allele	Cases		Controls		P value*
		Number	%	Number	%	
FOK I	FF	18	47	19	50	0.818
	Ff	17	45	16	42	
	Ff	3	8	3	8	
D543N	GG	14	37	3	8	**0.002**
	GA	23	60.5	35	92	
	AA	1	2.5	Nil	---	
3'UTR	TG/TG +/+	17	45	8	21	**0.028**
	TG/TG +/-	14	37	28	74	
	TG/TG -/-	7	18	2	5	
INT 4	GG	34	89	31	82	0.304
	GC	4	11	7	18	
	CC	Nil	---	Nil	---	

Note: *P value<0.05 is significant

Table 2: Gender distribution of gene polymorphism in the study subjects

Gene	Allele	Male (n=46)		Female (n=30)	
		Number	%	Number	%
FOK I	FF (37)	24	52	13	43.33
	Ff (33)	20	44	13	43.33
	ff (6)	2	4	4	13.33
D543N	GG (17)	13	28	4	13.3
	GA (58)	32	70	26	86.7

	AA (1)	1	2	Nil	--
3'UTR	TG/TG +/+ (25)	16	35	9	30.0
	TG/TG +/- (42)	24	52	18	60.0
	TG/TG -/- (9)	6	13	3	10.0
INT 4	GG (65)	41	89	24	80.0
	GC (11)	5	11	6	20.0
	CC (0)	Nil	--	Nil	--

Figure 1 (a-d): Disease activity with different allelic forms among Type 2 DM patients with TB

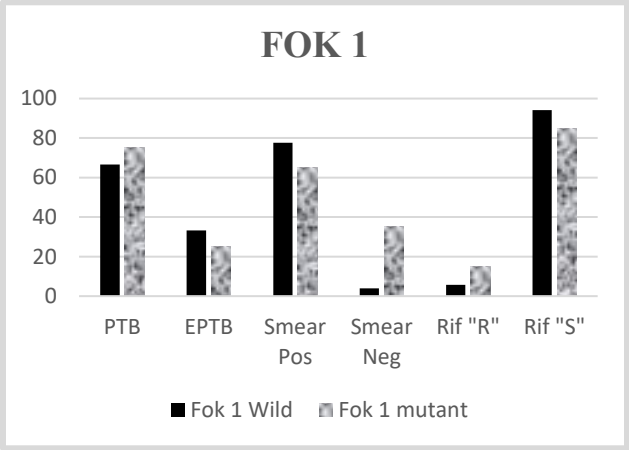


Figure 1(a)

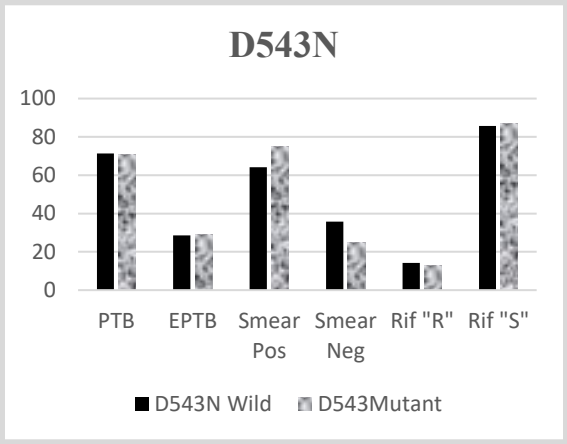


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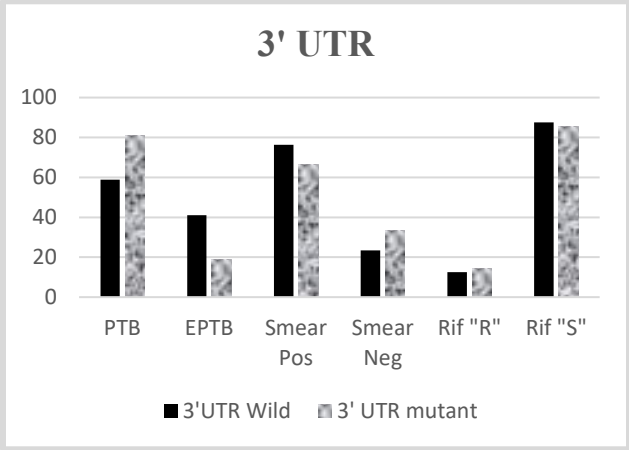


Figure 1(c)

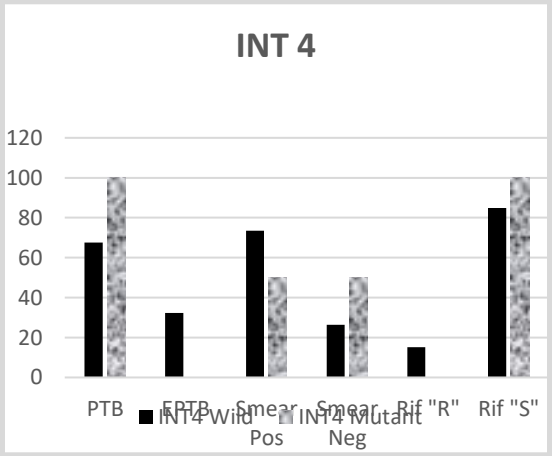


Figure 1(d)