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Research Article

Genetic Susceptibility of Inflammatory Genes in Household Contacts of Tuberculosis Patients with Diabetes Mellitus

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Abstract

Diabetes Mellitus (DM) is one of the prominent co-morbidities to Tuberculosis (TB) affecting one third of world's population. This study aims to establish association of IFN-γ(+874T>A, +5644 G/A), IL-1β (+3954C>T, -511C>T), IL-12 (+1188A>C, +1159C>A) and IL-10 (-1082A>G, -592C>A) cytokine genes in 130 TB patients with co-morbid DM (TBDM) and 125 Household Contacts (HHC) with respect to the healthy controls (HC, N=200). Genotyping was performed using specific primers by Amplification Refractory Mutation System (ARMS) and Refractory Fragment Length Polymorphism (RFLP) Polymerase Chain Reaction (PCR). The susceptible genotypes and alleles identified were: AT genotype of IFN-γ +897A>T in TBDM (p=0.027) and their HHC (p=0.027); G allele of IFN-γ +5644G>A in TBDM (p=0.011); TT genotype and T allele respectively at -511C>T variant in TBDM (p=0.008, p=0.02) and HHC (p=0.001, p=0.0001); C allele at IL-12 +1159 variant in HHC (p=0.023); AG genotype of IL-10 -1082 A>G in TBDM and HHC (p=0.0001); A/G and G/G genotypes (p=0.0001, p=0.0001) and G allele (p=0.013) in TBDM when compared to the HC. These associations might help in identifying the risk haplotype by developing prognostic markers enabling the high risk individuals to take precautionary measures thus avoiding/ delaying the disease onset.

Keywords: Cytokine Gene Variant; Single Nucleotide Polymorphisms; Disease Susceptibility; Association Studies; Haplotype; Diplotype

Highlights

- Positive association was observed with AT (IFN-γ), TT (IL-1β) and AG (IL-10) genotypes both in TBDM and HHC.
- T (IL-1β), G (IFN-γ) alleles were susceptible in TBDM and C allele of IL-12 +1159 C > A in HHC indicating their importance in the disease predisposition in HHC.
- Based on the MDR analysis, TBDM patients have shown strong synergistic interaction between IFN-γ+874 and IL-12 +1159C>A cytokine gene variants.

High risk combinations in the HHC might help in identifying the high risk individuals.

Abbreviations

IFN-γ: Interferon gamma; IL: Interleukin; TBDM: Tuberculosis patients with Diabetes Mellitus; HHC: Household Contacts; MDR: Multidimensionality Reduction; PPM: Public Private Mix; LD: Linkage Disequilibrium

Introduction

Diabetes Mellitus (DM), chronic non-communicable disease weakens the immune system making the individuals prone to develop Tuberculosis (TB); this DM and TB co-morbidity usually present a relatively poor disease progress and considered major public health problems. According to the World Diabetes Federation (WDF) and International Union against Tuberculosis and Lung Disease (IUATLD) the risk of developing TB is two to three times more in diabetics than in general population [1]. In 2020, an estimated 34.2 million new cases of diabetes (1 per 10 persons) were diagnosed among U.S. adults aged 18 years or older [2]. Diabetics have also shown increased reactivation of TB lesions and even aggravated hyperglycemia requiring higher doses of insulin. In DM, immune dysfunction is a predisposing factor and has serious implications in patient care in Indonesia where diabetes is strongly associated with TB. Co-morbidity of TB-DM exceeded that of TB-HIV (Human Immunodeficiency Virus) from retrospective data in a Mumbai population [3]. Various studies reported on the disease transmission in household contacts (high risk group for TB) from the patients and this observation carries far reaching importance regarding the prevention and control of TB. Contact investigations have a valuable impact on the health of the family and the community as a whole [4].

The disease manifestation depends on the balance between pro- and anti-inflammatory cytokines associated with disease protection and susceptibility respectively. As DM patients are at increased risk of developing TB, this susceptibility to mycobacterial infection is due to a defective Th1-cytokine response such as IFN-γ, IL-1β and IL-12. The Th2 response is activated by the production of anti-inflammatory cytokine like IL-10. The Single Nucleotide Polymorphisms (SNP) located at the promoter or the coding regions of cytokine genes result in their differential secretion and alteration in transcriptional activation [5]. IFN-γ, pro-inflammatory cytokine located on

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Table 1: Primer sequences of IFN-γ, IL-1β, IL-12 and IL-10 cytokine genes.

Cytokine gene	SNP property	Primer sequence	PCR Product size
IFN-γ +874A>T	Intron	**Common primer** 5′- TCAACAAAGCTGATACTCCA- 3′ **Forward primer** 5′-TCTTACAACACAAAATCAAATCA-3′ **Reverse primer** 5′-TTCTTACAACACAAAATCAAATCT-3′	ARMS 186 bp
IFN-γ +5644G>A	3′ UTR	**Control primer 1** 5′-TGC CAA GTG GAG CAC CCA A-3′ **Control primer 2** 5′-GCA TCT TGC TCT GTG CAG AT-3′ **Forward primer G** 5′-CCT TCC TAT TTC CTC CTT CG-3′ **Forward primer A** 5′ACC TTC CTA TTT CCT CCT TCA-3′ **Reverse primer** 5′ -GTC TAC AAC AGC ACC AGG C-3′	ARMS 289 bp
IL-1β +3954C>T	Intron	**Forward primer** 5′-GTT GTC ATC AGA CTT TGA CC-3′ **Reverse primer** 5′-TTC AGT TCA TAT GGA CCA GA -3′	RFLP-Taq I 249bp,135bp, 114bp
IL-1β -511 C>T	Promoter	**Forward primer** 5′-TGG CAT TGA TCT GGT TCA TC -3′, **Reverse primer** 5′-GTT TAG GAA TCT TCC CAC TT -3′	RFLP-Ava 1 305bp,190bp, 114bp
IL-12 +1188A>C	3′ UTR	**Common primer** 5′-ATCTTGGAGCGAATGGGCAT -3′, **Forward primer** 5′TTGTTTCAATGAGCATTTAGCATCT 3′ **Reverse primer** 5’TTGTTTCAATGAGCATTTAGCATCG -3’	ARMS 784 bp
IL-12 +1159C>A	Intron	**Forward primer** 5’ – ATTTGGAGGAAAAGTGGAAGA – 3’ **Reverse primer** 5’ – AATTTCATGTCCTTAGCCATA – 3’	RFLP- Taq 1 908bp,776bp, 140bp
IL-10 -1082A>G	Promoter	**Common primer** 5’CACTACTAAGGCTTCTTTGGGTA3‘ **Forward primer** 5’ ACACTACTAAGGCTTCTTTGGGTG3‘ **Reverse primer** 5’-GTAAGCTTCTGTGGCTGGAGTC-3’	ARMS 161bp
IL-10 -592C>A	Promoter	**Forward primer** 5’-CCTAGGTCACAGTGACGTGG -3‘ **Reverse primer** 5’-GCTAGTCAGGTAGTGCTCACC - 3’	RFLP- Rsa 1 411bp,236bp, 175bp

chromosome 12 regulates both innate and cell-mediated immune responses leading to mycobacterial pathogen clearance and tumor surveillance [6,7]. It has a direct effect on lymphoid cells like beta cells which may play a role in pathogenesis of Insulin Dependent Diabetes Mellitus (IDDM) by upregulating MHC class I, class II and adhesion molecules on pancreatic beta cells. Polymorphisms at +874 site (rs 2430561) were mostly associated with infectious diseases like TB and DM. A +5644 G/A (GenBank Accession no. M37265) variant described in the 3’ untranslated region (3’UTR) of the IFN-γ gene was reported to be mostly associated with brucellosis [8].

IL-1β is a pro-inflammatory cytokine located on chromosome 2 that augments inflammation and host defense. It is a potent mediator of chronic inflammation [9] and is expressed in excess at the diseased site in TB patients. It plays a major role in tissue remodeling, hence involved in the DM pathogenesis and its associated complications [10]. IL-1β gene variants at +3954C>T (rs 1143634) were mostly associated with TB and periodontal disease, -511C>T (rs 16944) variant with DM and TB.

IL-12 a pro-inflammatory cytokine situated on chromosome 5 is produced by macrophages and dendritic cells upon phagocytosis of *M.tb* bacilli. It is considered one of the key cytokines in host defense against *M.tb* infection [11]. IL-12p40 production influences T cell response and may therefore be important in diabetes pathogenesis [12]. Several intronic polymorphic sites were identified wherein a Taq 1 SNP at 1188A/C position (rs 3212227) [13] at the 3’ UTR was found [14] to be functional and associated with Insulin dependent diabetes mellitus [15] and TB. The C/A 1159 SNP (GenBank accession no. NM_002187) at the 3’UTR showed significant association in Type 1DM (T1DM) in the Caucasians [16].

IL-10 a potent anti-inflammatory cytokine positioned on chromosome 1 terminates the inflammatory signal in inflammatory cells. It down regulates T-cell mediated immune responses [17] and potentially blocks phagosome maturation in macrophages by bringing about *M.tb* persistence in the humans [18]. It prevents pancreatic beta cell destruction and brings about humoral immune responses, thus acting as a defensive mediator against T2DM and inflammation [19]. Human genetic studies have indicated an association of -1082A>G (rs 1800896) and -592C>A (rs 1800872) variants with pulmonary diseases and T2DM. These polymorphisms may affect

Table 2: PCR conditions of IFN-γ, IL-1β, IL-12 and IL-10 cytokine genes.

Cytokine	Initial Denaturation	Annealing	Extension	Final Extension	No. of cycles
IFN-γ +874 A>T	95°C -5m 95°C- 30s	60°C- 60s	72°C-60s	72°C-5 m	30
IFN-γ +5644G>A	94°C – 2m 96°C – 20s	64°C – 50s	72°C – 40s 96°C – 20s 61°C – 50s	72°C – 40s	30
IL-1β +3954C>T	95°C – 4m 95°C – 30s	59°C – 30s	72°C – 30s	72°C – 4m	33
IL-1β -511C>T	94°C – 5m 94°C – 1m	55°C – 1m	72°C – 1m	72°C – 7m	45
IL-10 -1082A>G	94°C-3m 94°C – 30s	60 °C-30 s	72°C-45 s	72°C-3m	31
IL-10 -592C>A	95°C - 5m 95°C – 1m	63°C – 1m	72°C – 1m	72°C – 10m	35
IL-12 +1188A>C	94°C – 3m 94°C-30 s	65°C-45 s	72°C-45s	72°C - 5m	35
IL-12 +1159C>A	95°C – 30s	60°C – 30	72°C – 30s	72°C – 7m	36

the transcriptional factor binding altering transcriptional variation [20]. The difference in the cytokine secretion profile may suggest that the immunological mechanism underlying the disease pathogenesis might be variable; however there are no association studies with these cytokine genes. Hence the present study was designed to identify the susceptible genotypes of IFN-γ (+874A>T, +5644G>A), IL-1β (+3954C>T, -511C>T), IL-12 (+1188A>C, +1159C>A) and IL-10 (-1082A>G, -592C>A) cytokine gene variants in tuberculosis patients with diabetes mellitus and their household contacts.

Materials and Methods

Study Selection

A total of 455 subjects including Tuberculosis patients with Diabetes mellitus (TBDM, N=130), their household contacts (HHC, N=125) and Healthy Controls (HC, N=200) were enrolled in the study. TBDM patients and their HHC were those who attended the PPM-DOTS (Public Private Mix-Directly Observed Treatment Short-course) free chest clinic, Tuberculosis Unit (TU) under the Revised National Tuberculosis Control Programme (RNTCP) implemented at Bhagwan Mahavir Medical Research Center (BMMRC). HCs without any family history of TB and DM were considered. The sputum microscopy for AFB was performed as per RNTCP with confirmed diagnosis of sputum, culture and chest X-ray in patients. Diabetes was confirmed based on blood sugar levels. Body Mass Index (BMI) was calculated in all the subjects and Tuberculin Skin Test (TST) was performed in TBDM and HHC but not in the HC. The study was approved by the institutional ethical committee. Information regarding ethnicity/races was not shown as the study was carried out in a limited population.

Presence of causes of secondary immunodeficiency such as HIV, transplants, Hypertension, malignancy, cardiac arrest, pregnant women and patients unwilling/unable to comply were excluded from the study. Structured questionnaires were documented for other demographic information like age, gender, BMI, BCG vaccination, sputum gradation, blood sugar levels and diabetes duration for all the subjects. Sputum gradation and diabetes duration were not assessed for the HC.

DNA Extraction

Two ml of venous blood was extracted from the subjects and collected in BD containers coated with an anticoagulant, Ethylene Diamine Tetra Acetic acid (EDTA). The genomic DNA was isolated using the Flexi gene kit (Qiagen) and quantified by spectrophotometer (Figures 1-4). Purity of DNA was checked using absorbance ratio 260 and 280 nm. A ratio of ~1.8 is generally accepted as "pure" for DNA.

Table 3: Demographic characteristics of TBDM, HHC and HC.

Epidemiological feature	TBDM (N=130) Mean±SD p value	HHC (N=125) Mean±SD p value	HC (N=200) Mean±SD p value
Age	45.25±11.85 **0.0001***	36.48±11.01 **0.0001***	30.17±9.3
Gender M/F	87(67)/43(33)	53(42)/72(58)	134(67)/66(33) **0.0001+**
BMI	34.7±15.7 ns	25.08±14.5 ns	24.9±4.45 ns
BCG Yes/No	74(57)/56(43)	64(51)/61(49)	177(89)/23(11) **0.0001+**
TST +ve/-ve	112 (86)/18 (14)	88 (70)/37 (30)	-
Blood sugar	202.8±83.9 **0.0001***	103.96±9.83 **0.037***	99.7±8.34

*Mean±SD independent samples t-test ; + x2 test; M/F-male/female; P/N-positive/negative; BMI- Body Mass Index; TBDM- Tuberculosis with Diabetes mellitus, HHC-Household Contacts of TBDM, HC-Healthy Controls.

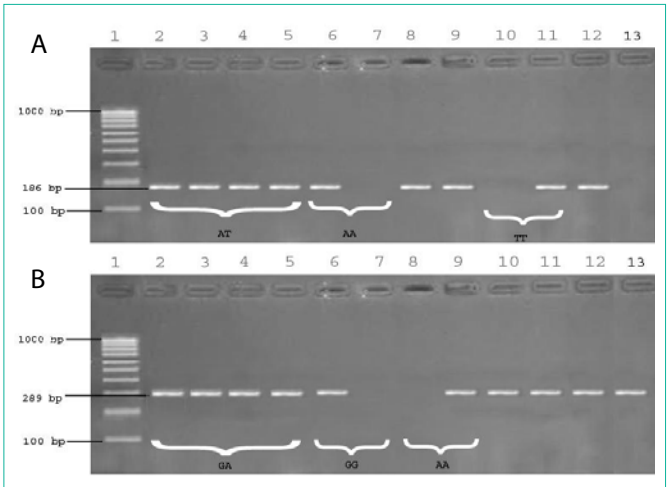

Figure 1: Agarose gel picture showing the amplified.
(a): ARMS-PCR products of IFN-γ +874A>T polymorphism
Lane 1- 100bp ladder; Lanes 2-3,4-5,8-9 – AT genotype; Lanes 6-7,12-13 – AA genotype; Lanes 10-11 – TT genotype.
(b): ARMS-PCR products of IFN-γ +5644G>A polymorphism.
Lane 1 – 100 bp ladder; Lanes 2-3,4-5,10-11,12-13 – GA genotype; Lanes 6-7 – GG genotype; Lanes 8-9 – AA genotype.

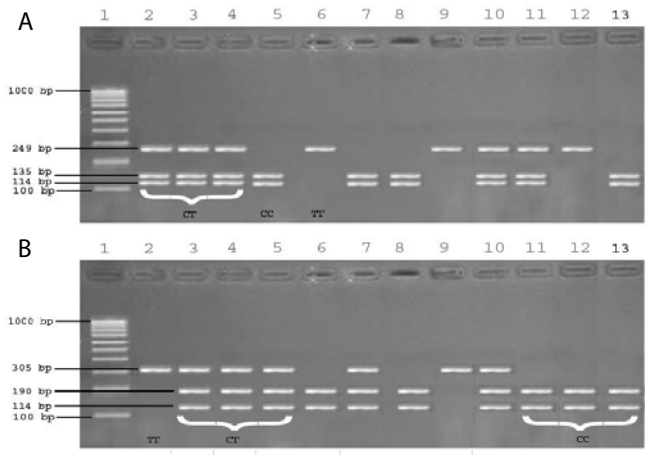

Figure 2: Agarose gel picture showing the amplified.
(a): PCR-RFLP products of IL-1β +3954 C>T polymorphism
Lane 1 – 100bp Ladder; Lanes 2,3,4,10,11 – CT genotype; Lanes 5,7,8,13 – CC genotype; Lanes 6,9,12 – TT genotype.
(b): PCR-RFLP products of IL-1β -511 C>T polymorphism.
Lane 1 – 100bp ladder; Lanes 3,4,5,7,10 – CT genotype; Lanes 6,8,11,12,13 – CC genotype; Lanes 2,9 – TT genotype.

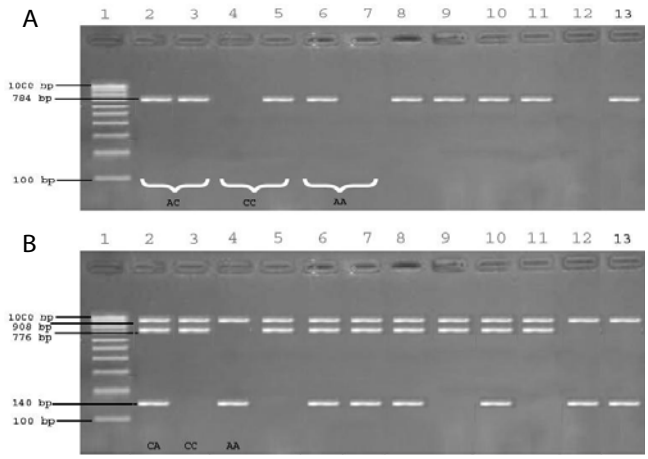

Figure 3: Agarose gel picture showing the amplified.
(a): ARMS-PCR products of IL-12 +1188 A>C polymorphism
Lane 1 – 100bp ladder; Lanes 2-3,8-9,10-11 – AC genotype; Lanes 4-5,12-13 – CC genotype; Lanes 6-7 – AA genotype.
(b): PCR-RFLP products of IL-12 +1159 C>A polymorphism.
Lane 1 – 100bp ladder; Lanes 2,6,7,8,10 – CA genotype; Lanes 3,5,9,11 – CC genotype; Lanes 4,12,13 – AA genotype.

SNP Selection and Genotyping

Candidate cytokine genes IFN-γ, IL-1β, IL-12 and IL-10 were selected based on their role in the pathogenesis of TB and DM and their SNPs were carried out byAmplification Refractory Mutation System (ARMS) and Restriction Fragment Length Polymorphism (RFLP). Primers were designed using PRIMER 3 software. The primer sequences, PCR (Polymerase Chain Reaction) conditions were shown (Table 1,2).

Statistical Analysis

All epidemiologic variables were determined using IBM SPSS Statistics 20 software, where student's t-test is used to evaluate continuous variables, and Ψ^2 test for categorical variables. Odds ratio (O.R), 95% Confidence Interval (CI) and AIC (Akaike's Information) were calculated using SNPstats. The gene-gene interaction for SNPs was analyzed by nonparametric Multifactor Dimensionality Reduction (MDR version 2.0 beta 8.4) analysis. Distribution of alleles and deviation of genotype frequencies were tested by using Hardy–Weinberg Equilibrium (HWE). P<0.05 was considered to be statistically significant for all the tests except HWE. Bonferroni's correction, an adjustment made to P values, was used to reduce the chances of obtaining false-positive results (p=0.0005). Linkage Disequilibrium was identified using Haploview software.

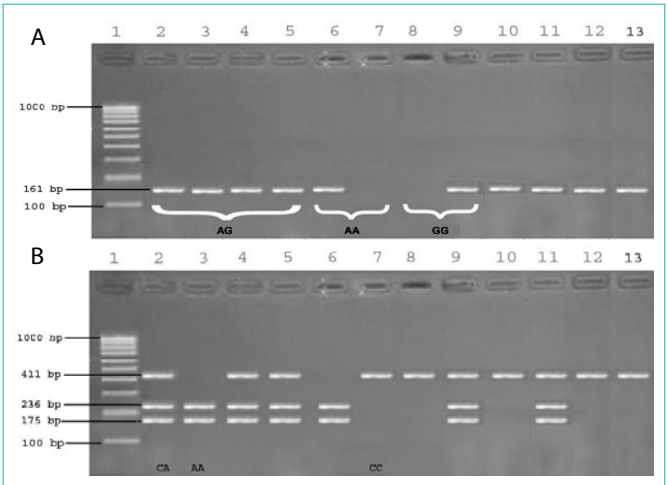

Figure 4: Agarose gel picture showing the amplified.
(a): ARMS-PCR products of IL-10 -1082 A>G polymorphism.
Lane 1 – 100bp ladder; Lanes 2-3,4-5,10-11,12-13 – GC genotype; Lanes 6-7 – GG genotype; Lanes 8-9 – CC genotype.
(b): PCR-RFLP products of IL-10 -592 C>A polymorphism.
Lane 1 – 100bp ladder; Lanes 2,4,5,9,11 – CA genotype; Lanes 3,6 – AA genotype; Lanes 7,8,10,12,13 – CC genotype.

Result

Demographic Features

The mean age of TBDM (87 males, 43 females), HHC (53 males, 72 females) and HC (134 males, 66 females) was 45.25±11.85, 36.48±11.01 and 30.17±9.3 with statistical significance in TBDM (p=0.0001) and HHC (p=0.0001) when compared to the HC. Males were predominant in TBDM and HCs while females in HHC (p=0.0001). The mean BMI was 34.7±15.7, 25.08±14.5 and 24.9±4.45 among TBDM, HHC and HC respectively without statistical significance. Significantly more number of BCG scar positive individuals were observed in HC (p=0.0001). BCG scar positive and negative individuals were almost evenly present in HHC and most of the TBDM and HHC were TST positive. The mean blood sugar levels were 202.8±83.9, 103.96±9.83 and 99.7±8.34 in TBDM, HHC and HC respectively with a statistical significance in TBDM (p=0.0001) when compared to HCs (Table 3).

Single Nucleotide Polymorphisms

IFN-γ (+874A>T, +5644G>A): At IFN-γ +874A>T position, positive association was observed with AT genotype both in TBDM *vs* HC (p=0.027, OR 1.7, 95% CI 1.06-2.73) and HHC *vs* HC (p=0.0001, OR 2.625, 95% CI 1.59-4.35). The AA genotype was protectively

Table 4: Genotype distribution of IFN-γ (+874A>T and +5644G>A) gene polymorphisms in TBDM patients, HHC and HC.

Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* OR		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
IFN-γ (+874A>T)									
A/A	26 (20)	22 (17.6)	64 (32)	0.531 (0.304-0.923)	0.023	0.454 (0.253-0.811)	0.007	1.17 (0.59-2.30)	0.741
A/T	80 (61.5)	89 (71.2)	97 (48.5)	1.7 (1.06-2.731)	0.027	2.625 (1.59-4.351)	0.0001	0.647 (0.37-1.13)	0.134
T/T	24 (18.5)	14 (11.2)	39 (19.5)	0.935 (0.51-1.703)	0.928	0.521 (0.25-1.05)	0.069	1.795 (0.84-3.88)	0.146
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* OR		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	(95% CI)	p value
A	132 (51)	133 (53)	225 (56)	0.802 (0.57-1.11)	0.175	0.884 (0.636-1.23)	0.466	0.91 (0.63-1.304)	0.645
T	128 (49)	117 (47)	175 (44)	1.247 (0.9-1.727)	0.175	1.131 (0.81-1.57)	0.466	1.102 (0.767-1.58)	0.645
IFN-γ (+5644G>A)									
Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* OR		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	(95% CI)	p value
A/A	46	47 (37.6)	76 (38)	0.893(0.55-1.451)	0.715	0.983 (0.60-1.60)	1	0.91 (0.53-1.56)	0.813
G/A	46 (35.4)	52 (41.6)	82 (41)	0.788 (0.49-1.28)	0.365	1.025 (0.64-1.66)	1	0.769 (0.45-1.32)	0.373
G/G	38 (29.2)	26 (20.8)	42 (21)	1.554 (0.91-2.67)	0.115	0.988 (0.55-1.772)	1	1.573 (0.85-2.91)	0.159
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* OR		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	(95% CI)	p value
A	138 (53)	146 (58)	234 (58)	0.657 (0.47-0.92)	0.011	0.816 (0.58-1.15)	0.24	0.81 (0.56-1.16)	0.263
G	122 (47)	104 (42)	136 (42)	1.521 (1.09-2.13)	0.011	1.226 (0.87 -1.72)	0.24	1.241 (0.86-1.79)	0.263

TBDM- Tuberculosis with Diabetes Mellitus, HHC- Household Contacts, HC-Healthy Controls, OR-Odds Ratio, CI- Confidence Interval.

Table 5: Genotype distribution of IL-1β (+3954C>T and -511C>T) gene polymorphisms in TBDM patients, TBDM HHC and HC.

Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
IL-1β +3954C>T									
C/C	63 (48.5)	54 (43.2)	86 (43)	1.246 (0.78-1.99)	0.39	1.008 (0.63-1.62)	1	1.236 (0.73-2.09)	0.473
C/T	64 (49.2)	58 (46.4)	94 (47)	1.093 (0.68-1.74)	0.78	0.976 (0.61-1.57)	1	1.12 (0.67-1.89)	0.743
T/T	3 (2.3)	13 (10.4)	20 (10)	0.213 (0.05-0.78)	0.014	1.045 (0.47-2.31)	1	0.204 (0.04-0.79)	0.016
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM HC OR (95% CI)	p value	HHC *vs* HC OR (95% CI)	p value	TBDM *vs* HHC OR (95% CI)	p value
C	190 (73)	178 (71)	266 (66)	1.367 (0.95-1.95)	0.085	1.245 (0.87-1.78)	0.226	1.098 (0.73-1.65)	0.708
T	70 (27)	72 (29)	134 (34)	0.731 (0.51-1.04)	0.085	0.803 (0.56-1.15)	0.226	0.911 (0.61-1.37)	0.708
IL-1β -511C>T									
Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
T/T	49 (37.7)	54 (43.2)	47 (23.5)	1.97 (1.18-3.28)	0.008	2.313 (1.39-3.84)	0.001	0.795 (0.47-1.35)	0.442
C/T	65 (50)	58 (46.4)	119 (59.5)	0.681 (0.43-1.09)	0.113	0.55 (0.34-0.88)	0.011	1.155 (0.68-1.95)	0.652
C/C	16 (12.3)	13 (10.4)	34 (17)	0.685 (0.34-1.36)	0.316	0.542 (0.26-1.121)	0.106	1.21 (0.52-2.81)	0.778
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	P value	OR (95% CI)	p value	OR (95% CI)	p value
T	163 (63)	166 (66)	213 (53)	1.475 (1.06-2.05)	0.02	1.735 (1.23-2.44)	0.0001	0.85 (0.58-1.24)	0.434
C	97 (37)	84(34)	187 (47)	0.678 (0.48-0.94)	0.02	0.576 (0.41-0.81)	0.0001	1.176 (0.80-1.72)	0.434

TBDM- Tuberculosis with Diabetes Mellitus, HHC- Household Contacts , HC-Healthy Controls, OR-Odds Ratio, CI- Confidence Interval.

associated in TBDM (p=0.023, OR 0.531, 95% CI 0.304-0.923) and HHC (p=0.007, OR 0.454, 95% CI 0.25-0.81) when compared to HC. None of the alleles have shown significant association with the disease both in TBDM and HHC. At +5644G>A variant, significant association was not observed with respect to the genotypes while the A allele (p=0.011, OR 0.657, 95% CI 0.47-0.92) was protectively associated and G allele (p=0.011, OR 1.521, 95% CI 1.09-2.13) was susceptible in the TBDM *vs* HC (Table 4). The genotype distribution

Table 6: Genotype distribution of IL-12 (+1188 A>C and +1159 C>A) gene polymorphisms in TBDM patients, HHC and HC.

Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
IL-12 +1188 A>C									
A/A	50 (38.5)	55 (44)	66 (33)	1.269 (0.78-2.06)	0.37	1.595 (0.98-2.59)	0.06	0.795 (0.47-1.35)	0.441
A/C	50 (38.5)	46 (36.8)	71 (35.5)	1.136 (0.70-1.84)	0.668	1.058 (0.65-1.73)	0.91	1.073 (0.63-1.84)	0.886
C/C	30 (23.1)	24 (19.2)	63 (31.5)	0.652 (0.38-1.11)	0.124	0.517 (0.29-0.91)	0.021	1.263 (0.66-2.41)	0.545
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
A	150 (58)	146 (58)	203 (51)	1.323 (0.95-1.84)	0.093	1.362 (0.98-1.89)	0.063	0.971 (0.67-1.40)	0.943
C	110 (42)	104 (52)	197 (49)	0.756 (0.54-1.05)	0.093	0.734 (0.53-1.02)	0.063	1.029 (0.71-1.49)	0.943
Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
IL-12 +1159C>A									
A/A	45 (34.6)	36 (28.8)	76 (38)	0.864 (0.53-1.41)	0.612	0.66 (0.39-1.09)	0.114	1.31 (0.75-2.30)	0.389
C/A	53 (40.8)	49 (39.2)	79 (39.5)	1.054 (0.66-1.69)	0.91	0.99 (0.61-1.60)	1	1.068 (0.63-1.82)	0.899
C/C	32 (24.6)	40 (32)	45 (22.5)	1.125 (0.65-1.95)	0.756	1.621 (0.95-2.76)	0.077	0.694 (0.39-1.24)	0.242
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI) 0.093	p value	OR (95% CI)	p value
A	143 (55)	121 (48)	231 (58)	0.894 (0.65-1.24)	0.52	0.686 (0.49-0.96)	0.023	1.303 (0.91-1.87)	0.161
C	117-(0.45)	129 (52)	169 (42)	1.118 (0.81-1.55)	0.52	1.457 (1.05-2.03)	0.023	0.767 (0.53-1.10)	0.161

TBDM- Tuberculosis with Diabetes mellitus, HHC- Household Contacts, HC-Healthy Controls, OR-Odds Ratio, CI- Confidence Interval.

Table 7: Genotype and allele distribution of IL-10 (-1082A>G and -592C>T) gene polymorphisms in TBDM patients, HHC and HC.

Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
IL-10 -1082A>G									
A/A	11 (8.5)	39 (31.2)	50 (25)	0.277 (0.13-.58)	0.001	1.36 (0.80-2.30)	0.275	0.204 (0.09-0.44)	0.0001
A/G	112 (86.2)	79 (63.2)	118 (59)	4.32 (2.36-7.99)	0.0001	1.193 (0.73-1.94)	0.524	3.623 (1.88-7.04)	0.0001
G/G	7 (5.4)	7 (5.6)	32 (16)	0.299 (0.12-0.74)	0.006	0.311 (0.12-0.77)	0.009	0.959 (0.29-3.16)	1
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
A	134 (52)	157 (63)	218 (55)	0.888 (0.64-1.23)	0.473	1.41 (1.0-1.97)	0.04	0.63 (0.44-0.91)	0.013
G	126 (48)	93 (37)	182 (46)	1.126 (0.81-1.56)	0.473	0.71 (0.51-0.99)	0.04	1.587 (1.09-2.29)	0.013
IL-10 -592C>T									
Genotype	TBDM N=130 (%)	HHC N=125 (%)	HC N=200 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
C/C	49 (37.7)	55 (44)	74 (37)	1.03 (0.64-1.67)	0.992	1.34 (0.83-2.16)	0.26	0.77 (0.45-1.31)	0.37
C/A	56 (43.1)	46 (36.8)	99 (49.5)	0.772 (0.48-1.23)	0.304	0.594 (0.37-0.96)	0.033	1.3 (0.76-2.22)	0.371
A/A	25 (19.2)	24 (19.2)	27 (13.5)	1.526 (0.81-2.88)	0.215	1.523 (0.79-2.89)	0.224	1.002 (0.51-1.96)	1
Allele	TBDM N=260 (%)	HHC N=250 (%)	HC N=400 (%)	TBDM *vs* HC		HHC *vs* HC		TBDM *vs* HHC	
				OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
C	154 (59)	156 (62)	247 (62)	0.9 (0.64-1.25)	0.568	1.028 (0.73-1.44)	0.934	0.875 (0.60-1.27)	0.521
A	106 (41)	94 (38)	153 (38)	1.111 (0.79-1.55)	0.568	0.973 (0.69-1.36)	0.934	1.142 (0.79-1.66)	0.521

TBDM- Tuberculosis with Diabetes mellitus, HHC- Household Contacts , HC-Healthy Controls, OR-Odds Ratio, CI- Confidence Interval

deviated from the HWE in TBDM and HHC at +874 but followed in HHC at +5644 position.

IL-1β (+3954C>T and -511 C>T): At +3954 C>T variant, the T/T genotype was protectively associated in TBDM *vs* HC (p=0.014) and TBDM *vs* HHC (p=0.016). The genotype distribution of TBDM patients deviated from HWE but HHC followed this trend. There was no statistically significant difference in the alleles both in TBDM and

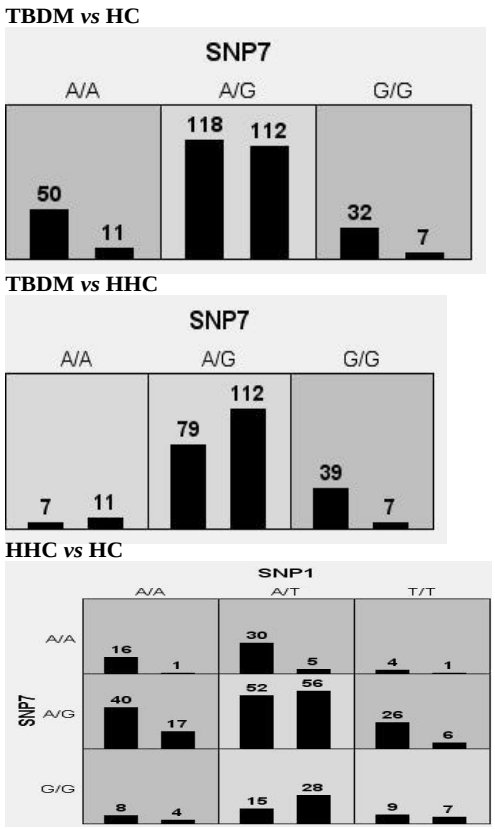

Figure 5: Bar diagram showing the gene –gene association by MDR analysis. SNP 1 IFN-γ+874T/A; SNP 7 IL-10 -1082A/G.
Dark boxes represent high risk combinations of genotypes. Light shaded boxes represent low-risk combinations. White boxes represent unclassified data.

their HHC. At -511C>T position, TT genotype with 1.97 and 2.313 times risk was observed in TBDM (p=0.008) and HHC (p=0.001) respectively when compared to HC. The T allele was positively associated with the disease (p=0.02; p=0.0001) with 1.475 times and 1.735 times risk in TBDM and HHC respectively when compared to the HCs. The C allele was protectively associated at p=0.02 in TBDM *vs* HC and p=0.0001 in HHC *vs* HC (Table 5). The genotype distribution was in accordance to HWE (p>0.05).

IL-12 (+1188A>C and +1159C>A): At variant +1188 A>C, protective association was observed with CC genotype in HHC vs. HC at p=0.021, while none of the alleles were significantly associated. At +1159 C>A variant significant association was not observed with respect to the genotypes while the A allele (p=0.023) was protectively associated and C allele (p=0.023) was positively associated with the disease in HHC vs. HC. The genotype frequency deviates from the HWE in TBDM and HHC (p<0.05) (Table 6).

IL-10 (+1082A>G and +592C>A): At -1082 A>G position, the AG genotype was positively associated in TBDM vs. HC (p=0.0001) and TBDM vs. HHC (p=0.0001) with 4.32 and 3.62 times risk respectively. Protective association was observed with AA genotype in TBDM vs. HC (p=0.001) and TBDM vs. HHC (p=0.0001) and with GG genotype in TBDM vs. HC (p=0.006) and TBDM vs. HHC (0.009). The G allele was positively associated (p=0.013) and A allele (p=0.013) was negatively associated in TBDM vs HHC. At -592 C>A position, significant association was not witnessed (Table 7). The genotype distribution follows HWE in TBDM but deviated in HHC in IL-10 variants. This deviation from HWE could be due to large number of heterozygotes within the patient category compared to HC.

MDR Analysis

When MDR analysis was carried out to study the genotype combinations for IFN-γ, IL-1β, IL-12 and IL-10 cytokine genes in TBDM, HHC and HC, high-risk and low-risk combinations were generated. In TBDM *vs* HC and TBDM *vs* HHC one-gene (IL-10 -1082A/G) interaction model and in HHC *vs* HC, two-gene (IFN-γ +874T/A and IL-10 -1082A/G) interaction model was considered best with a CV consistency of 10/10, testing balance accuracy of 0.6358, 0.6291 and 0.6555 in TBDM, HHC and HC respectively. In TBDM *vs* HC and TBDM *vs* HHC, A/A and G/G genotypes of IL-10 -1082A/G have shown high risk. In HHC *vs* HC, A/A, A/G and G/G genotypes of IL-10 -1082A/G have shown high risk with T/T, T/A and A/A genotypes of IFN-γ +874T/A (Figure 5).

Linkage Disequilibrium

Linkage Disequilibrium (LD) in population genetics is the non-random association of alleles at distinct loci i.e., alleles based on their frequencies are at different loci with statistical associations. In TBDM, HHC and HC, strong LD was not observed with any of the variants, however, moderate LD was observed between +5644G>Aand 1082A>G variant with a D' value of 0.729, LOD score of 1 and r^2 value of 0.184 in TBDM and between 874A>T and 1082A>G with a D'value 0.463, LOD 1.07 and r-squared 0.112 indicating that these

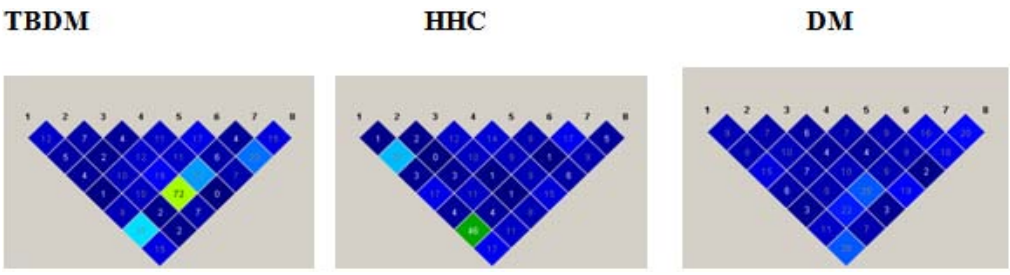

Figure 6: LD plot showing the cytokine markers IFN-γ (+874A>T; +5644G>A), IL-1(+3954C>T; -511C>T), IL-12 (+1188A>C; +1159C>A) and IL-10 (-1082A>G; -592C>A),) in TBDM, HHC and HC.
D'valuesare shown in each diamond.
Red, - strong LD, yellow, green -medium LD, white-no L.

variants may be associated with the disease (Figure 6). Haplotype block generation was performed using the algorithm by Gabriel et al implemented in the Haploview software which was also used for the initial association testing. When haplotypes were analyzed a total of 38 haplotypes were generated and out of which TACCCAGA haplotype was negatively associated at p=0.035 in TBDM *vs* HC. Its frequency was high in the HCs (0.021) when compared to the TBDM patients (0.004). Within various cytokine genes, specific risk haplotype was identified. Significant association was not observed with any of the haplotypes in HHC *vs* HC (data not shown).

Discussion

IFN-γ is the most important cytokine that provides resistance to mycobacterial diseases and most of its variants are involved with mycobacterium susceptibility. We selected polymorphism at +874 A>T position because the in vivo expression of this gene apart from its promoter is influenced by the generation of NF-kB site at +874 in the T allele [21]. In the present study, the AT genotype of IFN-γ +874 A>T polymorphism was positively associated in the TBDM and HHC. The G to A polymorphisms at +5644 position i.e., 3' UTR region of IFN-γ gene plays a pivotal role in mRNA stability, localization and translation efficiency regulation in Idiopathic pulmonary Fibrosis patients and healthy controls. In the current study, we have not observed any association of genotypes while G allele was susceptible in TBDM and HHC with respect to HCs. There were no reports on TBDM and TBDM HHC at these variants in IFN-γ cytokine gene. To our knowledge this was the first study with these variants in TBDM and their HHC.

IL-1β is expressed in an inactive precursor form (pro-IL-1β) in a variety of immune cells and then cleaved by caspase 1 to an active form via an inflammasome and controls *M.tb* infection. IL-1 β was reported to play an important role in the TB pathogenesis both in mice and the human subjects [22]. The IL-1β +3954 polymorphism was found to be protective in TBDM and HHC in this study. Studies were reported in Chronic Periodontitis subjects with DM wherein a statistically significant difference was not observed in genotype distribution when compared to the controls in a Kerala population [23] which was dissimilar to our study. Related observation was reported in periodontitis patients where no statistically significant difference was seen between patients and HCs [24]. The IL-1β -511 C>T promoter polymorphism has been shown to affect IL-1β processing and protein production and in the current study TT genotype and T allele were found to be positively associated both in TBDM and HHC.

IL-12 plays a crucial role in immunity to intracellular bacteria like Mycobacteria. An association was found between A to C mutation at position 1188 in the 3ÚTR of the IL-12B gene leading to decreased protein secretion and it might be a cause of common predisposition to Th1 mediated infectious diseases like TB and Salmonellosis. TBDM patients have not shown any association with this polymorphism unlike in the HHC where CC genotype was protectively associated. At 1159C>A position, genotype association was not observed, however C allele was positively associated with the disease in HHC.

IL-10 is an important multifunctional cytokine that displays immunomodulatory effects and immune stimulation. It brings about increased anti mycobacterial activity by converting human dendritic cells into macrophage like cells. The SNPs at IL-10 -1082A>G found on the promoter region exhibit strong effect on IL-10 gene transcription. The A/G genotype frequency was high in the TBDM patients when compared to the other genotypes unlike in the Mexican population [25], Brazilian population [26] and in a population from Egypt [27-29] where G/G genotype frequency was presented with greater frequency. The zygosity status determines the inheritance of the cytokine gene production profile in patients that might impact the disease outcome. When IL-10 polymorphisms were studied at -592C>A position, our study did not report any association of this polymorphism in TBDM and HHC.

Based on the association studies, positive association was observed with AT genotype of IFN-γ +874A>T, TT genotype and T allele of IL-1β-511C>T and AG genotype of IL-10 -1082 A>G both in TBDM and HHC, G allele of +5644G>A variant was susceptible in the TBDM and C allele of IL-12 +1159 C>A was positively associated with the disease in HHC indicating their importance in the disease predisposition in HHC. Three of the HHCs of these patients developed the disease had susceptible genotypes, TT of IFN-γ +874A>T and AG genotype of IL-10-1082A>G. Based on the MDR analysis, TBDM patients have shown strong synergistic interaction between IFN-γ +874 and IL-12 +1159C>A cytokine gene variants signifying that they are the high risk combinations affecting the signaling mechanism leading to disease. The presence of high risk combinations in the household contacts may help in identifying the individuals at risk of disease predisposition. Further studies in other ethnic groups are needed to fully validate these results to disclose the potential function of these SNPs.

Author Contribution

Conceived and designed the experiments: SG, Performed the experiments: MP, Analyzed the data: MP, SG. Contributed reagents/materials/analysis tools: SG. Drafted the manuscript: MP SG. Revising it critically for important intellectual content: SG MP.

Conflict of Interest Declaration

The authors declare that there are no conflicts of interest.

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