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Variations in macrophage migration inhibitory factor gene are not associated with visceral leishmaniasis in India

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ABSTRACT

Background: The host genetic factors play important role in determining the outcome of visceral leishmaniasis (VL). Macrophage migration inhibitory factor (MIF) is an important host cytokine, which is a key regulator of innate immune system. Genetic variants in *MIF* gene have been found to be associated with several inflammatory and infectious diseases. Role of MIF is well documented in leishmaniasis diseases, including Indian visceral leishmaniasis, where elevated level of serum MIF has been associated with VL phenotypes. However, there was no genetic study to correlate *MIF* variants in VL, therefore, we aimed to study the possible association of three reported *MIF* gene variants −794 CATT, −173G > C and non-coding RNA gene *LOC284889* in Indian VL phenotype.
Methods: Study subjects comprised of 214 VL patients along with ethnically and demographically matched 220 controls from VL endemic regions of Bihar state in India.
Results: We found no significant difference between cases and controls in allelic, genotypic and haplotype frequency of the markers analysed [-794 CATT repeats ($\chi2 = 0.86$; p = 0.35; OR = 0.85; 95% CI = 0.61–1.19); −173 G > C polymorphism ($\chi2 = 1.11$; p = 0.29; OR = 0.83; 95% CI = 0.59–1.16); and *LOC284889* ($\chi2 = 0.78$; p = 0.37; OR = 0.86; 95% CI = 0.61–1.20)].
Conclusion: Since we did not find any significant differences between case and control groups, we conclude that sequencing of complete *MIF* gene and extensive study on innate and adaptive immunity genes may help in identifying genetic variations that are associated with VL susceptibility/resistance among Indians.

Introduction

Leishmaniasis is a group of diseases, which exists in four clinical forms — cutaneous, muco-cutaneous, visceral, and post kala-azar dermal leishmaniasis (PKDL). All four forms are predominant in tropical regions and considered as neglected tropical disease. The most severe form of leishmanial diseases is visceral leishmaniasis (VL) or kala-azar which leads to mortality, if untreated [1]. *Leishmania donovani* is the protozoan parasite that causes visceral leishmaniasis in Indian subcontinent (India, Nepal, and Bangladesh), which represents 67% of world VL diseases and approximately 150 million people are at risk of developing VL [2–4]. In India, people of the Bihar state, who are with low socio-economic status, are the most affected by this disease [2–5].

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Leishmania parasites have dimorphic life cycle between sand fly vector and human host [6]. The life cycle of parasite starts once it enters into the human blood stream through infected sand fly biting. After entering inside the host, parasite invades host immune system through various strategies. The primary target of the parasite is a macrophage, a part of the innate immune system [6,7]. Simultaneously, parasitic infection induces the T lymphocytes to trigger the host innate immune response by secreting the immune molecules to encounter the invading pathogen. Of these, cytokines play important role in controlling the immune response by regulating the balance between humoral and cell mediated immunity [8,9]. The complex interaction between Th1 and Th2 cytokines can generate protective immunities against intracellular parasites [10,11]. It was established earlier that genetic susceptibility and resistance is regulated by various immunological cytokines/factors [12,13]. Genetic variations in cytokine secreting genes can deregulate the cytokine production, which may lead to a non-specific response during disease pathogenesis. Further, it has also been shown that single nucleotide polymorphisms (SNPs) are the most frequently occurring genetic variations responsible for varied phenotypic expression as well as differential disease susceptibility and responses to drugs [14,15]. Therefore, identification and validation of genetic variations in cytokine producing genes will help in understanding the genetic basis of disease resistance and susceptibility in the endemic region [16].

Macrophage migration inhibitory factor (MIF) cytokine is secreted by lymphocytes, macrophages, dendritic cells, neutrophils, and pituitary cells in humans [17]. MIF cytokine is found in various cell types and is released upon different stimuli (cytokine stimulation and variations on glucocorticoid levels) during infection process. MIF acts by producing tumor-necrosis-factor-α (TNF-α), interleukin-1 (IL-1), nitric oxide (NO) in humans [18]. MIF secretion is responsible for increased expression of TLRs and adhesion molecules in macrophages as it triggers CD44/CD74 receptor complex along with CXCR2 and CXCR4 chemokine receptors. MIF mediates its activity by three independent pathways; (a) signaling through JAB1/CSN5 pathway; (b) receptor-mediated pathway and (c) through enzymatic catalysis [19–23].

Human *MIF* gene has been mapped on to 22q11.23 [24]. It is evolutionary conserved; having high degree of homology with rats, gerbils, cattles, suggesting that it may exert some important biological functions. Structurally it contains three exons, two introns, and several putative transcription factor binding sites [25]. The exact role of these transcription factors and how they control the expression of *MIF,* is not well studied. However, two variants of *MIF*, *MIF-794 CATT (5–8)* repeats (rs145871794; 5′UTR variant) and *–173 G > C* (rs755622; promoter variant) were found to be associated with various inflammatory and infectious diseases [26]. The promoter polymorphism *-173*C* allele increase the susceptibility to systemic-onset juvenile idiopathic arthritis and juvenile idiopathic arthritis in UK patients [27]. In Japan, patients with ulcerative colitis showed increased severity with -173C/C genotype [28]. The 5′UTR *CATT*5* allele reduces the severity of rheumatoid arthritis in US patients [29]. The *CATT*6, 7* and *8* repeats were correlated with increased parasitemia and severe malarial anemia in Zambian and Kenyan children [30,31]. Additionally *CATT*7/-173*C* haplotype was also found to increase the susceptibility of inflammatory polyarthritis in UK patients [32]. Interestingly, an uncharacterized non-coding RNA gene *(LOC284889)* (rs34383331) present within 1kb downstream of *MIF* gene was reported to be highly associated with severe case of malaria in Indian population [26].

Infection with various species of *Leishmania* parasites results in higher expression of serum MIF levels, which favours immune, as well as inflammatory responses, thereby helps in elimination of parasites and complex regulation of the antimicrobial activity of infected cells [33]. Studies on animal models established that *MIF* gene-deficient mice are susceptible to cutaneous *Leishmania major* infection [34]. Additionally, it was also proved that purified recombinant MIF activates murine macrophages to kill *L. major* [35]. Kozaci et al. revealed that human patients with acute cutaneous leishmaniasis caused by *Leishmania tropica* showed high MIF levels compared to healthy subjects [36].

MIF is produced in the lymph node of a mouse infected with *L. major* and *in-vivo* administration of MIF (recombinant) reduces the severity of infection [37]. MIF plays a critical role in mediating host resistance to leishmaniasis. An *in vitro* study on Indian population/patients demonstrated that *Leishmania* infected T cells can potentially generate more MIF levels [18]. Host genetics play a significant role in VL susceptibility or disease severity in Indian endemic region [38–40]. The genetic etiology for the VL susceptibility in Indian population shows a different pattern compared to rest of the world populations [38]. It is important to study susceptibility/resistance alleles among Indian populations, which would provide a distribution pattern of susceptibility/resistance allele. Interestingly, several other innate immunity components that were studied in the same endemic region were found to be important for VL [38–40]. Although several studies have provided the evidence for the importance of *MIF* expressions in various diseases including leishmaniasis, none of the studies tried to correlate *MIF* gene polymorphisms with VL phenotype, particularly among the Indian population. Hence, the aim of this study is to find the possible role of *MIF* variants in Indian VL phenotype.

Table 1A
Demographic factors of studied subjects.

	Sample size	Mean age (year) ± SD	Male:female
Controls	220	35.3 ± 16.2	128:91
Cases	214	28.7 ± 16.7	119:95

Material and methods

Study subject and sampling

Present study was designed as case-control study (ethnicity, age group, sex and geographical location). We have made multiple field visits in VL endemic regions,VL patients and ethnically matched healthy controls were recruited from villages located within a radius of ~100 km from the city of Muzaffarpur, covering the border area of district of Vaishali, Patna, Samastipur and East Champaran in Bihar state. Blood samples (about 5.0 ml) were collected from a total of 434 unrelated subjects, including 214 VL patients along with ethnically and demographically matched 220 controls, after obtaining written informed consent. This study was approved by the Institutional Ethical Committee (IEC) of CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India.

Cases were ascertained through presence of typical clinical features of VL in the medical records issued from hospitals in endemic regions. The major clinical features include, fever with rigors and chills, splenomegaly, serologically rk39 positive and most importantly parasitological positive in splenic aspirates of cases [41,42]. We matched cumulatively VL patients and ethnicity. The control subjects were healthy and never affected with any infectious diseases in the region (visceral leishmaniasis, tuberculosis and malaria or others). Further, family history was confirmed for VL up to three generations (among first, second and third degree relatives). The mean age of cases and controls was 28.7 ± 16.7; 35.3 ± 16.2, respectively. The male: female ratio in cases was 119:95; while in controls it was 129:91 (Table 1A).

Table 1B
Association analysis[a] using logistical regression with age and gender as correction factor.

	Case	Control	P-value
Subjects	215	220	–
Age (mean ± standard deviation) (t-test)	34.24 ± 17.32	28.73 ± 16.44	0.000725
Gender (M/F) (χ^2-test)	119/96	128/91	0.58
CATT-repeats (55/56/66/67/77) (GLM)	9/66/102/34/4	14/66/110/27/3	0.69
1202G > C (CC/GC/GG) (GLM)	13/65/137	10/61/149	0.64
2889T > A (AA/TA/TT) (GLM)	10/71/134	10/63/147	0.62

[a] Generalized linear model (GLM): ANOVA (GLM (Group vs. (variation + age + gender))).

Genomic DNA extraction and primer designing

Genomic DNA was extracted from whole blood using the modified protocol described by Thangaraj et al. [43]. The reference sequence was retrieved from ENSEMBL and the target specific primer pairs were designed using the Primer-BLAST described elsewhere [38]. Primer sequences used for amplification and sequencing are given in Table 2.

Genetic analysis

The Short Tandem Repeat [*-794 CATT (5–8)*] and two SNP (-173 G > C and rs34383331 T > A) were amplified using Takara Emerald Amp GT PCR kit on GeneAmp 9700 (Applied Biosystem) following manufacturer's protocol. Thermal cycling condition for amplification were: initial denaturation at 94 °C for 5 min, followed by 32 cycles of 30 s at 94 °C (denaturation), 25 s at 59 °C –62 °C (annealing) and 1 min at 72 °C (extension), followed by a final extension of 7 min at 72 °C (Table 2). After checking on 2% agarose gel, 5.0 μl of amplicons were cleaned up by treating with 2.0 μl of Exo-SAP (USB, Affymetrix) at 37 °C for 20 min followed by enzyme deactivation at 80 °C for 15 min. After Exo-Sap treatment, 1.0 μl of the purified PCR products were used as templates for sequencing, using the BigDye terminator (v.3.1) cycle sequencing kit (Applied Biosystems) and ABI 3730XL DNA sequencer, according to the manufacturer's instructions. Genomic differences were noted by comparing the sequences of subjects with the reference sequence using AutoAssembler software (Applied Biosystem). Observed variations were validated by resequencing of subset of samples.

Statistical analysis

The allele and genotype frequencies were calculated by simple gene counting and Expectation Maximum (EM) algorithm. Further,significance of deviations from Hardy-Weinberg equilibrium was tested using Plink v1.07 and Linkage disequilibrium (LD) analysis using Haploview v4.2 software. The allele and genotype distribution were calculated by chi-square test in different sample sets using online tool "VassarStats" [44]. The haplotype association analysis for multiallelic markers was performed using WHAP v2.09 package [45]. In all analysis, a two tailed p-value less than 0.05 were considered significant. Magnitude of association was expressed as odds ratio with a 95% confidence interval (CI).

For association analysis using logistical regression with age and gender as correction factor, we used 'glm' function of R, to perform regression analysis and ANOVA to explore overall effect of variation.

Results

Comparison of genotype and allele frequency for MIF *variants*

The genotype and allele frequency distribution for *–794 CATT (5–7)* polymorphisms for both case and control groups are as follows; genotype frequency ($\chi2 = 1.42$; p = 0.49) and allele frequency ($\chi2 = 0.86$; p = 0.35; OR = 85; 95% CI = 0.61–1.19) respectively. Furthermore, comparison was made for individual genotype (*CATT5/5, CATT5/6, CATT6/6, CATT6/7 and CATT7/7*) and allele (*CATT*5*, *CATT*6* and *CATT*7*) frequency distributions. We have observed only three alleles for the locus *-794CATT* (*CATT*5, CATT*6* and *CATT*7* allele) (Table 3 and Table 4). This study also recorded same distribution pattern for *CATT6/6* genotype in both cases and controls (case = 103 (48.1%); control = 110 (50%); p = 0.70; OR = 0.92; 95%CI = 0.63–1.35) and for *CATT*6* allele (case = 271 (63.3%); control = 286 (65%); p = 0.62; OR = 0.92; 95% CI = 0.70–1.22) with no association in Indian VL patients.

The genotype frequency ($\chi2 = 1.03$; p = 0.59) and allele frequency ($\chi2 = 1.11$; p = 0.29; OR = 0.83; 95% CI = 0.59–1.16) for the promoter polymorphism –173G > C did not show significant difference between cases and controls. Similarly, the genotype ($\chi2 = 0.82$; p = 0.66) and allele ($\chi2 = 0.78$; p = 0.37; OR = 0.86; 95% CI = 0.61-1.20) frequencies distribution for the rs34383331 polymorphism (T > A) also did not shows significant difference between cases and controls (Table 3 and Fig. 1).

Comparison of diplotypes and haplotype for MIF *variants*

By constructing three diplotypes among (a) -794CATT5-7 and -173G > C (b) -794CATT5-7 and rs34383331 (c) -173G > C and rs34383331 and haplotype among all three polymorphism also shows no statistical significance for the distribution pattern of any constructs (Table 5). Hence, only the haplotype distribution values have been mentioned for study and not for any of the diplotypes. Frequency of the haplotype 6GT was the maximum in both the groups [Controls = 127 (58.6%); Cases = 118 (55.0%); p = 0.43; OR = 0.84; 95%CI = 0.57–1.23], whereas 7GA haplotype, observed only in control group [3 (1.4%; p = 0.11); OR = 0.00] is the minimum (Table 5). Control samples in all the three variants were in Hardy Weinberg equilibrium.

Association analysis using logistical regression with age and gender as correction factor

We noticed that age distribution is significantly different between case and control (t-test p-value = 0.000725) while gender distribution was not significantly different ($\chi2$ p-value = 0.579). Hence, we performed association analysis in 2 different ways, (1) using logistical regression with age and gender as correction factor, and (2) by categorizing the subjects into 3 age categories (<18, 18–50 and >50 years) and performing logistical regression with age and gender as correction factor in each categories. We did not find any significant differences among case and control groups (Tables 1B and 1C).

Discussion

MIF is one of the important immunological molecule, involved in immunity regulation at the disease stage (reviewed in Box 1) [46]. MIF controls *Leishmania* burden by increasing macrophage activation through enhancement of TNF and NO production [18,47]. Since modern humans migrated out of Africa to rest of the world,

Table 1C
Association analysis by categorizing the subjects into 3 age categories (<18, 18–50 and >50 years) and performing logistical regression with age and gender as correction factor in each categories.

		Case	Control	Significance
Category 1 (age < 18 years)[a]	Subjects	71	38	–
	Age (mean ± standard deviation) (t-test)	12.13 ± 2.46	12.28 ± 4.10	0.83
	Gender (M/F) (χ^2-test)	37/34	20/18	1
	CATT-repeats (55/56/66/67/77) (GLM)	2/25/33/9/2	0/17/15/6/1	0.56
	1202G > C (CC/GC/GG) (GLM)	4/24/1943	1/8/1930	0.24
	2889T > A (AA/TA/TT) (GLM)	4/30/1937	1/12/2026	0.35
Category 2 (age < 18–50 years)[a]	Subjects	121	140	–
	Age (mean ± standard deviation) (t-test)	32.59 ± 10.54	32.47 ± 10.09	0.92
	Gender (M/F) (χ^2-test)	65/56	81/59	0.59
	CATT-repeats (55/56/66/67/77) (GLM)	5/35/56/23/2	10/38/73/17/2	0.46
	1202G > C (CC/GC/GG) (GLM)	9/34/78	6/39/95	0.54
	2889T > A (AA/TA/TT) (GLM)	5/37/79	7/40/93	0.9
Category 3 (age > 50 years)[a]	Subjects	23	41	–
	Age (mean ± standard deviation) (t-test)	59.65 ± 5.65	61.17 ± 6.92	0.35
	Gender (M/F) (χ^2-test)	17/06	27/14	0.7
	CATT-repeats (55/56/66/67/77) (GLM)	2/6/13/2/0	4/11/22/4/0	0.99
	1202G > C (CC/GC/GG) (GLM)	0/07/16	3/14/2024	0.22
	2889T > A (AA/TA/TT) (GLM)	1/4/2018	2/11/2028	0.67

[a] Generalized linear model (GLM): Group vs. (variation + age + gender).

Table 2
Primer sequences and conditions for the PCR of the*MIF* variants.

Variants			
Primer (5′–3′)	rs145871794 (-794,CATT(5–8))	rs755622 (-173,G > C)	rs34383331 (ncRNA gene LOC284889)
Forward sequence	GCCTGTGATCCAGTTGCTGCC	GGTACAGGGGCTCAGTGCGT	CCCAGAGCCACTCACTGCCA
Reverse sequence	CCTCAGGGAAGCCACACCCT	GTCCCGCCTTTTGTGACGCC	GTACTGCGCGGGATGACCGA
Annealing temperature (°C)	59 °C	62 °C	62 °C

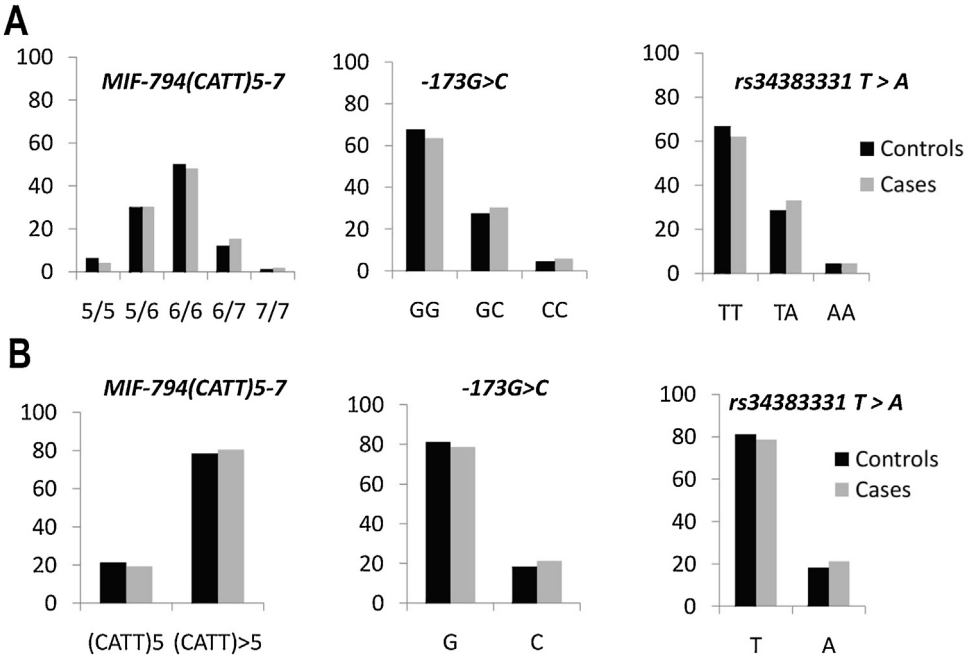

Fig. 1. Distribution of *MIF* variants in case–control groups. (A) Genotype and (B) allele.

they might have encountered various pathogens. As a result, the frequency of most protective alleles might have increased in the human population [48]. In this context, this study was designed to find the possible association of *MIF* variants with Indian VL phenotype, which might possibly contribute to the genetic basis of VL resistance/susceptibility. Although *in vitro* study demonstrated that Indian VL patients potentially generate less MIF levels, which is attributed to the lower number of T cells expressing CD2 cells [18]. However, after treatment, up regulation of $CD2^+$ T cells with pronounced MIF response was observed [18]. A study shows that increased/decreased level of MIF decreases lesion size and mediates leishmanicidal effects of IFN-γ on macrophage [47]. Evidences also suggest that MIF is a key regulator for a number of diseases, including rheumatoid arthritis (RA), septic shock, delayed-type hypersensitivity and cancer [49], which confirms its diversified role in health and disease across the world population. We have per-

Table 3
Genotype and allele frequency distribution of *MIF* variants in case-control groups.

Group	Genotype (%)			Genotype comparison		Allele (%)		Allele comparison		
				χ^2_2	P value			χ^2_1	P value	OR (95% CI)
rs145871794, (CATT*5,6,7)	(CATT)6,6/7,7	(CATT)5,6/6,7	(CATT)5/5			(CATT)5	(CATT)>5			
Controls (n = 220)	113 (51.3)	93 (42.2)	14 (6.3)			94 (21.3)	346 (78.6)			
Cases (n = 214)	106 (49.5)	99 (46.2)	9 (4.2)	1.42	0.49 (NS)	83 (19.3)	357 (83.4)	0.86	0.35 (NS)	0.85 (0.61–1.19)
rs755622, G > C	GG	GC	CC			G	C			
Controls (n = 220)	149 (67.7)	61 (27.7)	10 (4.5)			359 (81.5)	81 (18.4)			
Cases (n = 214)	136 (63.5)	65 (30.3)	13 (6.0)	1.03	0.59 (NS)	337 (78.7)	91 (21.2)	1.11	0.29 (NS)	0.83 (0.59–1.16)
rs34383331, T > A	TT	TA	AA			T	A			
Controls (n = 220)	147 (66.8)	63 (28.6)	10 (4.5)			357 (81.1)	83 (18.8)			
Cases (n = 214)	133 (62.1)	71 (33.1)	10 (4.6)	0.82	0.66 (NS)	337 (78.7)	91 (21.2)	0.78	0.37 (NS)	0.86 (0.61–1.20)

NS — not significant; *Tri-allelic CATT repeats were down coded as bi-allelic (CATT) 5 repeats and (CATT) >5 repeats; $: Data were compared between controls and each case group using chi square test; @2 × 3 contingency chi square test for genotype and @2 × 2 contingency chi square test for allele; Values in parenthesis have been rounded off.

Table 4
Comparison of individual *MIF*-794 CATT repeat genotype and allele frequencies among case-control group.

Genotype	Controls	Cases	Controls vs cases[a]	
	(n = 220)	(n = 214)	P-Value	OR (95% CI)
5/5	14 (6.36)	9 (4.2)	0.39	0.64 (0.27–1.52)
5/6	66 (30.0)	65 (30.3)	1	1.01 (0.671.53)
6/6	110 (50.0)	103 (48.1)	0.7	0.92 (0.63–1.35)
6/7	27 (12.2)	33 (15.4)	0.4	1.30 (0.75–2.25)
7/7	3 (12.2)	4 (1.8)	0.72	1.37 (0.30–6.23)
Allele	Controls (n = 440)	Cases (n = 428)	P-Value	OR (95% CI)
5	94 (21.3)	83 (19.3)	0.5	0.88 (0.63–1.23)
6	286 (65.0)	271 (63.3)	0.62	0.92 (0.70–1.22)
7	33 (7.5)	41 (9.5)	0.27	1.30 (0.80–2.10)
Grouped				
5	94 (21.3)	83 (19.3)	0.5	0.88 (0.63–1.23)
>5	346 (78.6)	345 (80.6)	0.5	1.12 (0.81–1.57)

NS — not significant; values in parenthesis have been rounded off.
[a] Fisher's two tailed exact test.

Table 5
Distribution and comparison of *MIF* haplotypes (*MIF*-794CATT, rs755622G > C and rs34383331T > A) in case-control group.

Haplotype	Control (%)	Case (%)	Case vs control[a]	
	Total = 213	Total = 213	P value	OR (95% CI)
6GT	127 (58.6)	118 (55.0)	0.43	0.84 (0.57–1.23)
5GT	37 (17.6)	36 (16.9)	0.99	0.96 (0.58–1.60)
6CA	17 (8.4)	20 (9.4)	0.73	1.19 (0.60–2.34)
7CA	8 (3.9)	11 (5.3)	0.63	1.39 (0.54–3.54)
6GA	4 (2.0)	8 (3.7)	0.38	2.03 (0.60–6.87)
6CT	7 (3.3)	8 (3.7)	1	1.14 (0.40–3.22)
7GT	5 (2.4)	7 (3.6)	0.77	1.41 (0.44–4.52)
5CA	5 (2.5)	5 (2.4)	1	1.00 (0.28–3.50)
7GA	3 (1.4)	0 (0.0)	0.11	0.0 (0.0–0.0)

Values in parenthesis have been rounded off.
[a] Fisher's two tailed exact test.

formed logistical regression with age and gender as a correction factor in each category (Tables 1B and 1C). We want to rule out any possibility of influence on the mean age or even the gender matching of cases and controls (mean age is 28.7 *vs* 35.3 years, respectively). Our analysis (logistic regression), shows that age distribution is significantly different between case and control (t-test p-value = 0.000725) while gender distribution was not significantly different ($\chi 2$ p-value = 0.579). Hence, we performed association analysis in 2 different ways, (1B) using logistical regression with age and gender as correction factor, and (1C) by categorizing the subjects into 3 age categories (<18, 18–50 and >50 years) and performing logistical regression with age and gender as a correction factor in each category. We used 'glm' function of R, to perform regression analysis and ANOVA to explore overall effect of variation. Moreover, we did not find any significant differences among case and control groups of Indian VL (Tables 1B and 1C). Our study clearly signifies that all three investigated *MIF* polymorphisms were not associated with VL in India and plausible explanation underlies in probable complex regulation of *MIF* and unique Indian population structure.

Although, we did not find association of studied *MIF* variation in VL among Indians, case-control and functional study in the Iraq shows that *MIF* polymorphism (*MIF-173G > C*) association with cutaneous leishmaniasis [50]. In Southeast Brazil, study on American tegumentary leishmaniasis (ATL) which is caused by *Leishmania* (*Viannia*) *braziliensis*, suggest that the lower levels of MIF produced

Box 1: Key facts of macrophage inhibitory factor (MIF) pathway and regulation.

A- **MIF pathway**- one of the mechanisms of mediating MIF biological activity is by binding to CD74 and phosphorylating the extracellular signal/Kinase 1 (ERK1)/ERK2 protein. MIF is also known to bind JUN-activation domain-binding protein 1 (JAB 1), preventing JAB1-induced activation of JUN and JAB1-induced degradation of the cell–proliferation inhibitor KIP1 thereby leading to cell cycle arrest and apoptosis [46].

B- MIF innate immune cells regulation-MIF upregulating TLR-4 receptor on macrophages, which in turn allows rapid recognition of endotoxin containing bacteria, which promotes the production of cytokines like MIF, nitric oxides (NO) and other mediators. [46].

C- MIF immunosuppressive effects- MIF regulategluco-corticoids at transcriptional and posttranscriptional levels. It inhibits the gluco-corticoids mediated induction of inhibitor of nuclear factor-κB (IκB), synthesis and destabilization of messenger RNA, and overrides the gluco-corticoides mediated inhibition of PLA2 activity and arachidonic acid production and iNOS (inducible nitric oxide synthase) [46].

by *MIF* -173C carriers could influence the host–*Leishmania* interaction, favoring infection and disease progression [51]. The above studies show differential pattern of host response to *Leishmania* infection compared to Indian population with respect to -173G > C and *-173*C* allele, [27,28,32,50,51].

Although *MIF* variants-*794CATT5-7* is not significantly associated in this study, but *CATT5/5* genotype and *CATT*5* allele were associated with decreased malaria severity in another population [25]. We did not find any significant difference between the distribution of *CATT5/5* genotype (p = 0.39; OR = 0.64; 95%CI = 0.27-1.52) and *CATT*5* allele (p = 0.50; OR = 0.88; 95%CI = 0.63–1.23) frequency (Table 4), whereas selective advantage of *CATT*5* allele in malaria patients is well documented [25]. The frequency of *CATT6/6* genotype and *CATT*6* allele was maximum in Indian malaria patients, while our study shows no association of *CATT*6* allele with VL [26]. Similarly, SNP rs34383331 (T > A), which showed an association among Indian malaria patients, did not show any association among Indian VL patients.

Down coding of MIF *-794CATT repeats*

Although previous studies on different population have reported four alleles of *-794CATT* repeats, we observed only three alleles (*CATT*5, CATT*6,* and *CATT*7* allele). Previous studies in various infectious and inflammatory diseases have demonstrated that the presence of more than five *CATT* repeats increases the susceptibility and severity of malaria [28–31]. Hence, the tri-allelic STR (*-794CATT5-7* repeat) has been down-coded as bi-allelic marker; *CATT*5* repeats as one allele and *>CATT*5* repeats (*CATT*6* and *CATT*7*) as another allele (Table 3 and Table 4). Comparison of diplotypes and haplotype for *MIF* variants indicates no statistical significance. Interestingly, the frequency of the haplotype 6GT was the maximum in both the groups, whereas 7GA haplotype, observed only in the control group with lowest frequency (Table 5).

All the control samples were in Hardy–Weinberg equilibrium for the sequenced variants. Linkage disequilibrium (LD) pattern showed strong LD pattern between rs755622 *(-173G > C)* and rs34383331 (T > A) variation. It has already been established that Indian VL patients displayed lower MIF levels [18], however in this study; VL phenotype is not significantly associated with reported *MIF* variants. This result may also be a possible outcome of complex immune regulation in VL, where apart from hosts' *MIF* variants; other gene(s)/pathway(s) determine the disease resistance and susceptibility. Possibly other genes and pathways, which are related to *MIF* genes, may be playing important role in over expression of MIF cytokine in Indian VL. Further, a crucial study on other downstream factors might reveal the MIF severity mechanism in Indian VL.

Xu et al. demonstrated that oral administration of attenuated *Salmonella* transfected plasmids encoding the murine *MIF* and the *TNF-α*, enhanced the resistance of BALB/c mice infected with *L. major*. Resistance in mice was associated with high iNOS expression compared to the other plasmids encoding *MIF*, *TNF-α* or *IFNγ* genes alone. Therefore, MIF plays a protective role against leishmaniasis, likely by synergizing with other inflammatory cytokines such as TNF- α *via* the induction of iNOS expression [47].

Additionally, a study on *Salmonella typhimurium* shows that *MIF* deficient mice unable to control the growth of pathogens and succumbed to infection. Further, this study suggests that *MIF* deficient mice show the presence of higher bacterial count due to reduced plasma levels of the cytokines TNF, IL-12, and IFN-γ [46]. In another study, it was found that P-4 antigen vaccinated BALB/c mice were resistant to *L. pifanoi* infection. Resistance was conferred by $CD4^+$ T cell producing MIF, TNF-α or IFN-γ but not IL-4 [47]. Study on *MIF* knockout (KO) mice revealed that susceptibility to *L. major* infection is due to impaired macrophage leishmanicidal activity rather than deregulation of Th1/Th2 responses [34]. It was also found that after anti-leishmanicidal treatment, VL patient showed higher numbers of $CD2^+$ T cells, indicating a possible role for $CD2^+$ T cells and its ability to produce MIF in the immune response to VL [18].

All these findings suggested that MIF play a very important role against leishmaniasis. MIF acts as an immune regulator and kills *Leishmania* parasites directly or indirectly with other cytokines and nitric oxide production. However further *in-vivo* studies are required to exactly pinpoint the mechanism of leishmanicidal responses after *MIF* induction. Our earlier studies on *IL4* variations have demonstrated complex regulation of IL4 cytokine in Indian population [52,53]. Finally, It is important to understand the genetic etiology of *MIF* susceptibility in the Indian population, since India is one of the major foci of VL [38]. Study of genetic susceptibility/resistance may help in understanding the role of *MIF* variants in VL. We have earlier established that the Indian populations are genetically unique [54,55] and follow strict endogamy, at least for the last 2000 years [56], hence we expect variation in allele frequency between different Indian populations. Therefore, *MIF* variation among Indian populations are also expected to be different compared to rest of the world populations (Iraq and Brazil) [50,51] due to their unique origin and the practice of strict endogamy [52–56]. Hence, we have selected representative samples of case and control from same vicinity of the VL endemic regions.

The present study suggests that onset of leishmaniasis may not be solely dependent on *MIF* alone but the other genes involved in MIF pathway (TLR4, IFN-γ, NO *etc.*) might also be involved in upregulation or downregulation of MIF. Additionally, several other factors including activation of Th1 and Th2 cytokines and Treg cell also play a crucial role in the regulation of immune mechanism in VL patients [38]. Role of $CD4^+$ and $CD8^+$ T cells, in *Leishmania* protection is well documented in *L. major* and *L. donovani* respectively [57,58]. The study shows that skin-resident $CD4^+$ T cells protect against *L. major* by recruiting and activating inflammatory monocytes [57]. Further, we recommend genotyping of additional host genetic factors in ethnically different populations for the better understanding of associated genetic risk factors in leishmania infection. Host genetics and dynamics of host-pathogen interactions could have played a significant role in bringing various outcome in Indian VL susceptibility [38–40,52]. Our study established fur-

ther research on other downstream immune molecules of *MIF* for understanding more on host regulation of Indian VL.

Conclusion

We did not find any significant difference between case and control groups, as revealed by regression analysis and ANOVA. This study confirms the differential role exhibited by host genetic factors in response to infection of VL protozoan parasite among Indian populations. Further, our study provides a better understanding of how genetic variations on different ethnic background play a role in disease phenotypes. Hence, we suggest that sequencing of complete *MIF* gene and extensive study on innate and adaptive immunity genes may be required for identifying variations that are associated with VL susceptibility/resistance among Indians.

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Competing interests

None declared.

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