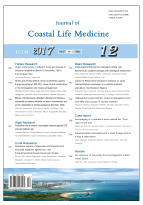
Journal of Coastal Life Medicine

journal homepage: www.jclmm.com



Original article https://doi.org/10.12980/jclm.5.2017J7-151

Haptoglobin polymorphism, plasma haptoglobin level and ABO blood group in leprosy patients

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ARTICLE INFO

Article history:
Received 9 Oct 2017
Received in revised form 15 Nov 2017
Accepted 18 Nov 2017
Available online xxx Nov 2017

Keywords:
ABO blood group
Haptoglobin concentration
Haptoglobin polymorphism
Leprosy

ABSTRACT

Objective: To analyze haptoglobin polymorphism, plasma haptoglobin concentration, and ABO blood groups associated with leprosy.
Methods: Blood groups were determined using monoclonal anti-A and anti-B. Haptoglobin was genotyped by PCR; plasma haptoglobin concentration was measured by ELISA. Data were analyzed by SPSS version 21 and *P*-values ⩽ 0.05 were considered as statistically significant.
Results: ABO blood groups in leprosy patients were found to have no significant difference compared to controls and the general population ($P > 0.05$). The data showed a lower frequency of Hp^{1-1} (14.6%) and higher frequency of Hp^{2-2} (25.6%) in leprosy patients compared to healthy controls (23.1% and 19.8%, respectively), without significant association ($P = 0.315$). The mean of haptoglobin concentration was higher in leprosy patients [(1.32 ± 0.70) mg/mL] than in healthy controls [(1.17 ± 0.67) mg/mL] ($P = 0.160$). The mean (1.44 mg/mL) and median (1.37 mg/mL) values of haptoglobin concentration were significantly higher in male leprosy patients than in male healthy controls (1.11 mg/mL and 1.11 mg/mL, respectively) ($P = 0.018$). Independent sample *t*-test and One-way ANOVA analysis also indicated significant mean elevation of Hp along leprosy spectrum and bacterial index ($P < 0.05$).
Conclusions: In conclusion, the study revealed absence of influence of Hp polymorphism and ABO blood groups on leprosy occurrence; however, plasma haptoglobin concentration elevates in leprosy patients and is significantly associated with leprosy spectrum and bacterial loads in patients.

1. Introduction

Leprosy is a human chronic infectious disease caused by obligate intracellular bacteria, *Mycobacterium leprae*, which affects skin and peripheral nerves. The disease presents itself in different clinico-pathological forms depending on the immune status of the host[1], thus its clinico-pathological features correlate with local cytokine patterns and the nature of T-cell responses[1].

Human haptoglobin (Hp), an acute phase hemoglobin binding protein, is encoded by a gene located on chromosome 16 (16q22), having two common co-dominant alleles (Hp1 and Hp2) [2] that bring about three phenotypic classes: Hp^{1-1}, Hp^{1-2} and Hp^{2-2}. Haptoglobin gene is principally expressed in the liver by hepatocytes[3], induced by cytokines such as interleukin-6 (IL-6), IL-1 and tumor necrosis factor-α (TNF-α)[4]. Structurally, it has four chains: 2α light chains and 2β heavy chains[5], and following its secretion, circulates as a linear dimer, or as a linear polymer, or as a cyclic polymer. In healthy human adults, plasma Hp level varies between 0.38 g/L and 2.08 g/L[6], but the range may elevate in response to inflammation, bacterial infection, tissue destruction and malignant diseases[4,7]. Besides its hemoglobin scavenging role, Hp has many biological functions such as oxidative stress prevention[8], anti-oxidant function[9], nitric oxide related function[10], anti-bacterial activities[6], renal damage prevention[6,8], anti-inflammatory functions[8], inhibition of prostaglandin synthesis[11], and immune modulation[8,12]. The modulation function of Hp both

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The journal implements double-blind peer review practiced by specially invited international editorial board members.

in cellular and humoral immune systems[12,13] plays a key role during *M. leprae* infection[14] including leukocyte recruitment and migration, modulation of cytokine patterns following microbial infection and tissue repair[15]. Moreover, the role of Hp to balance the two polar immunological T-helper cell responses (Th1 and Th2) by promoting a dominant Th1 cellular response[13,16] determines the clinic-pathological features of leprosy[1]. In leprosy, tuberculoid (TT) patients are characterized by Th1-dominant cytokine response with low intracellular bacilli load, and lepromatous (LL) patients are characterized by Th2-dominant cytokine response with abundant bacilli[1,17,18]. The borderline leprosy patients show unstable immune response with intermediate intracellular bacillary load[1].

Biological functions of Hp including anti-oxidant, scavenging, anti-inflammatory, and immune-regulatory properties vary based on Hp genotypes[4]; and the genetic polymorphism of Hp has been shown to influence the course of a number of inflammatory and immune functions. Although innate and adaptive immune responses play key roles during *M. leprae* infection and Hp immune modulator property is important to regulate these two lines of defenses, little is known about role of Hp polymorphism and its concentration in the pathogenesis or clinical course of leprosy. The presently reported case-control study aimed to assess the Hp gene polymorphism, plasma Hp concentration, and ABO blood group relation among leprosy patients from ALERT referral hospital in Ethiopia.

2. Materials and methods

2.1. Subjects

The study participants were new case of leprosy patients who came for treatment at All Africa Leprosy Tuberculosis Rehabilitation Research Training Center Hospital (ALERT) from different regions of Ethiopia. The patients were consecutively enrolled from the beginning of August 2013 to the end of October 2013, and composed of 82 leprosy patients (49 males and 33 females). The control subjects were recruited based on their socio-demographic backgrounds and consisted of 91 healthy subjects (60 males and 31 females).

2.2. Leprosy diagnosis

Leprosy patients were diagnosed based on clinical parameters which were involved in dermato-neurological examinations and bacteriological investigation of acid fast bacilli (AFB) in the slit skin smears. Then they were classified according to Ridley-Jopling leprosy classification system and World Health Organization (WHO).

2.3. Blood grouping/DNA extraction

About 2–3 mL venous blood samples were collected using tubes containing anti-coagulant (EDTA) and the ABO blood groups were examined by antigen-antibody agglutination test using slide method and commercially available anti-A and anti-B monoclonal antibodies (Tulip Diagnostics, India). Genomic DNA was extracted from peripheral blood leukocytes by standard non-organic (proteinase K and salt out) extraction procedure[19].

2.4. ELISA

Plasma haptoglobin level was quantified by ELISA method using human Hp ELISA kit (ab108856 haptoglobulin human ELISA kit) (synthesized by Abcam, UK). The assays were conducted following the instructions provided by the manufacturer.

2.5. Hp genotyping

The Hp1 and Hp2 alleles were amplified by PCR as the method previously described by Koch *et al*.[20] and the oligo-nucleotide primers were synthesized by Applied Bio-systems (Kenya). A total of 20 μL PCR reaction mixture was used per reaction. Each reaction mixture consisted of 10 μL HotStarTaq Master Mix Kit (providing a final concentration of 1.5 mmol/L $MgCl_2$ in the final reaction mix, 2.5 IU HotStarTaq DNAP, 1× PCR buffer, and 200 μmol/L of each dNTP) (synthesized by Qiagen), 1 μL each of the two (0.5 pmol/μL) primers, template DNA (≈ 1 μg/50 μL), and 7 μL distilled water. Each reaction mixture was set to be amplified at different PCR amplification programs based on the primer combinations in the mixtures. The first PCR reaction mixture containing primers A and B was set for touchdown PCR program under the following conditions: 95 ˚C for 15 min (95 ˚C for 30 s, 69 ˚C–1 ˚C with each cycle for 45 s, and 72 ˚C for 1 min and 30 s) for 15 cycles, 95 ˚C 30 s, 55 ˚C for 45 s, and 72 ˚C for 1 min and 30 s for 35 cycles, and 72 ˚C for 7 min. The PCR involving C and D primers used standard conditions: 95 ˚C for 15 min (95 ˚C for 45 s, 69 ˚C for 2 min) for 35 cycles and 72 ˚C for 7 min. PCR was conducted by thermal cycler TC-512.

2.6. Gel electrophoresis

The PCR amplicons of the two reactions (Reaction-1 containing primers A and B; Reaction-2 containing primers C and D) were combined and differentiated by electrophoresis using 1% agarose gel, 1× TAE buffer, ethidum bromide, loading dye, and 100 V for 45 min.

2.7. Statistical analysis

Allele frequencies were calculated under the assumption of Hardy–Weinberg equilibrium and expressed as percentages. *Chi*-square test was used to compare observed allelic and genotypic frequency distributions of the blood group and haptoglobin. Shapiro-Wilk test and an inspection of the Skewness and Kurtosis measures and standard errors as well as visual inspections of their histogram,

normal Q-Q plots, and pox plots were used to test for the normal distribution of continuous data. In order to check for all possible differences among the groups, ANOVA was used. Comparisons between groups were made using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant and the analyses were performed using SPSS version 21 software. The OD data were converted into concentrations with Graphpad prism 6 Demo Software (www.graphpad.com).

2.8. Ethical consideration

The protocol was approved by the Armauer Hansen Research Institute (AHRI/ALERT) Ethical Review Committee and the Ethical Review Committee of College of Natural Science, Addis Ababa University. Moreover, full consent forms were signed by the subjects after the aim of the study was clearly explained to them.

3. Results

3.1. Haptoglobin genotype/ABO blood group

The distribution of ABO blood phenotype in leprosy patients was: A (30.5%), B (22.0%), AB (6.0%), and O (41.5%). These proportions were found to have no significant difference as compared to healthy controls (Pearson *Chi*-square test; $X^2(3) = 0.601$, $P = 0.896$, 2-sided). The phenotypic distribution between studied populations and the comparison with the general population (data obtained from Ethiopian National Red Cross[21]) is presented in Table 1.

The frequency of the three Hp genotypes was in Hardy-Weinberg equilibrium ($P > 0.05$) and the distribution in patients was: Hp^{1-1} (14.6%), Hp^{1-2} (59.8%), and Hp^{2-2} (25.6%). There was low percentage of Hp^{1-1} and high percentage of Hp^{2-2} in leprosy patients as compared to healthy controls (Table 1); however, the data showed lack of significant difference between the groups ($P = 0.315$). For leprosy patients, the frequency of Hp1 was 44.5% and Hp2 was 55.5%; while in controls the frequencies of the two alleles were 51.6% and 48.4%, respectively (Table 1). Hp2 allele frequency was higher in leprosy patients, although the difference lacked significance ($P = 0.185$); and the odd ratio (OR) of developing leprosy in individuals having Hp1 was lower than individuals had Hp2 allele (Hp1/Hp2, OR = 0.751, 95% *CI*: 0.492–1.147).

Figure 1 indicates the graphic comparison of genotypic and allelic distribution of Hpin Paucibacillary (PB) and Multibacillary (MB) leprosy types along with healthy controls. Unlike its frequency in healthy controls, the frequency of Hp^{2-2} in PB

Table 1
ABO blood group, Hp genotype and allele frequency in cases, controls and population.

	ABO blood group				Hp genotype			Hp allele	
	A	B	AB	O	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	Hp1	Hp2
Case [*N* (%)]	25 (30.5)	18 (22.0)	5 (6.0)	34 (41.5)	12 (14.6)	49 (59.8)	21 (25.6)	73 (44.5)	91 (55.5)
Control [*N* (%)]	29 (31.9)	16 (17.6)	5 (5.5)	41 (45.1)	21 (23.1)	52 (57.1)	18 (19.8)	94 (51.6)	88 (48.4)
Population [*N* (%)]	4414 (30.9)	3522 (24.7)	381 (2.7)	5969 (41.8)	ND	ND	ND	ND	ND
Total [*N* (%)]	4469 (30.9)	3556 (24.6)	391 (2.7)	6044 (41.8)	33 (19.1)	101 (58.4)	39 (22.5)	167 (48.3)	179 (51.7)
X^2 (*df*), *P*-value	8.524 (6), 0.202				2.312 (2), 0.315			1.759 (1), 0.185	

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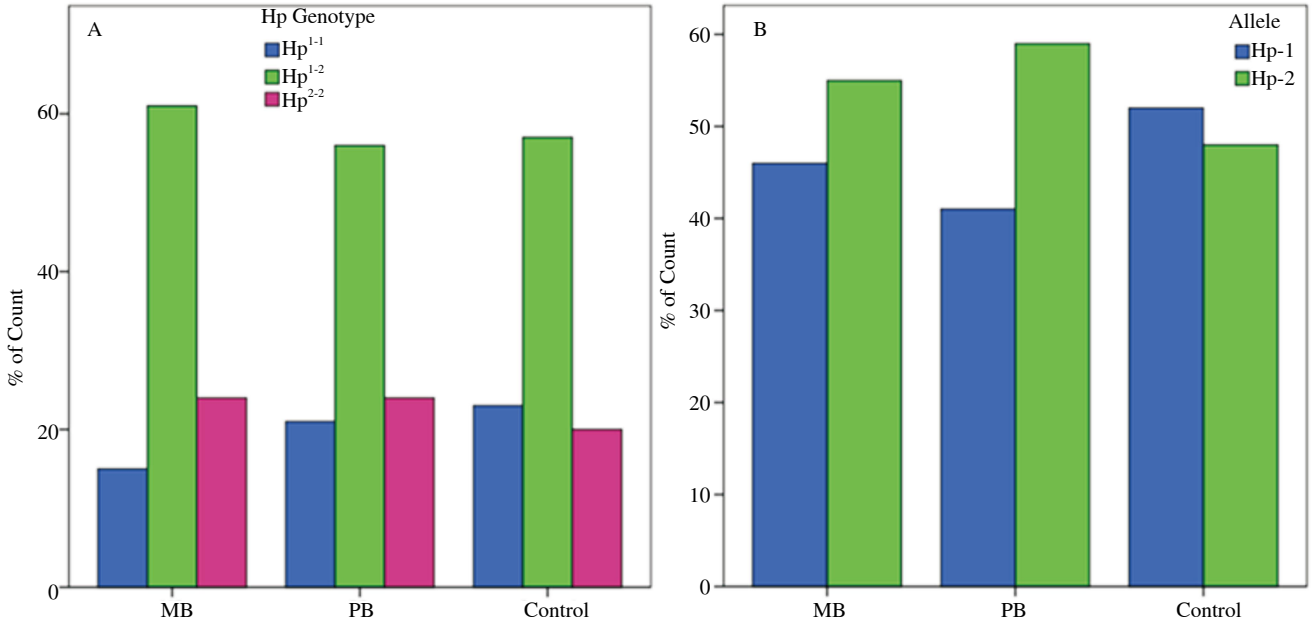

Figure 1. Polymorphism of Hp in Paucibacillary (PB) and Multibacillary (MB) patients. A: Genotype distribution; B: Allele distribution.

and MB (23.8% and 24.2%, respectively) was greater than the frequency of HP^{1-1}, and this was also true for Hp2 frequency in PB and MB (59.4% and 54.5%, respectively).

3.2. Haptoglobin concentration

The age range of studied subjects was 18–54; in each age category the proportions did not differ significantly from each other at 0.05 level: 18–24 (30 cases and 35 controls), 25–34 (26 cases and 31 controls), 35–44 (13 each), 45–54 (13 cases and 12 controls). In every age category, plasma Hp concentration was higher in leprosy patients compared to healthy controls, but not significant (the minimum *P*-value was 0.238). Haptoglobin plasma concentration of leprosy patients and healthy controls was approximately normally distributed with means of (1.32 ± 0.70) mg/mL and (1.17 ± 0.67) mg/mL, respectively, and there was no significant difference between them ($P = 0.160$). However, the mean and median were significantly higher in male leprosy patients (1.44 mg/mL and 1.37 mg/mL, respectively) than in male healthy controls (1.11 mg/mL and 1.11 mg/mL, respectively) (independent sample *t*-test, $P = 0.018$); but there was no significant mean and median difference between female leprosy patients and female healthy controls (independent sample *t*-test, $P = 0.351$). Independent sample *t*-test and One way ANOVA analysis also indicated significant mean elevation of Hp along leprosy spectrum and bacterial index ($P < 0.05$). Table 2 shows the comparison of Hp plasma level in relation to study participants category, leprosy spectrum, leprosy reaction type, and bacterial index.

Table 2

Hp plasma level comparison by study participants category, gender, leprosy spectrum, bacterial index, and reaction type.

		N	Mean ± SD	Range		*t*-test	ANOVA test
				Min	Max	*P*-vale	*P*-value
Category	Case	82	1.32 ± 0.70	0.10	3.91	0.16	…
	Control	91	1.17 ± 0.67	0.04	2.81		
Female	Case	33	1.14 ± 0.58	0.14	2.60	0.351	...
	Control	31	1.29 ± 0.68	0.31	2.76		
Male	Case	49	1.44 ± 0.76	0.10	3.91	0.018	
	Control	60	1.11 ± 0.66	0.04	2.81		
Leprosy spectrum	LL	22	1.81 ± 0.71	0.52	3.91	…	< 001
	BL	21	1.32 ± 0.60	0.40	2.60	0.018[a]	
	BB	24	1.02 ± 0.66	0.14	2.48	< 001[a]	
	BT	15	1.08 ± 0.52	0.10	2.26	0.002[a]	
Bacterial index (BI)	BI = 0	32	0.97 ± 0.57	0.10	2.48	< 001[b]	< 001
	BI = 1	7	1.00 ± 0.47	0.14	1.70	0.01[b]	
	BI = 2	3	1.52 ± 1.04	0.52	2.60	0.561[b]	
	BI = 3	9	1.58 ± 0.63	0.74	2.58	0.439[b]	
	BI = 4	9	1.32 ± 0.55	0.40	2.05	0.082[b]	
	BI > 4	22	1.80 ± 0.71	0.75	3.91	…	
Leprosy reaction	Type I	14	1.05 ± 0.75	0.19	2.60	0.082[c]	0.16
	Type II	8	1.62 ± 0.64	0.52	2.58	…	
	No reaction	60	1.34 ± 0.69	0.10	3.91	0.279[c]	

LL: Lepromatous leprosy; LB: Borderline lepromatous leprosy; BB: Borderline leprosy; BT: Borderline tuberculoid leprosy.

$X^{a,b,c}$: *P*-value comparisons of LL *v.s* non-LL; BI greater than 4 *v.s* BI less than 4; Type II *v.s* non-Type II reactions.

The mean, minimum (min), and maximum (max) values are given in mg/mL.

The mean of Hp^{1-1}plasma level in leprosy patients [(1.69 ± 0.81) mg/mL] was greater than the mean of Hp^{1-1} plasma level in healthy controls [(1.34 ± 0.59) mg/mL] (independent sample *t*-test, $P = 0.159$). Similarly, the mean of Hp^{1-2} plasma level [(1.42 ± 0.63) mg/mL] in patients was greater than the mean of Hp^{1-2} plasma level [(1.23 ± 0.68) mg/mL] in healthy controls (independent sample *t*-test, $P = 0.149$). However, the mean of Hp^{2-2} plasma concentration was approximately the same both in leprosy patients and healthy controls [(0.88 ± 0.62) mg/mL and (0.82 ± 0.64) mg/mL, respectively] (independent sample *t*-test, $P = 0.770$). Figure 2 shows the comparison of Hp plasma level in leprosy patients and healthy controls by Hp genotype.

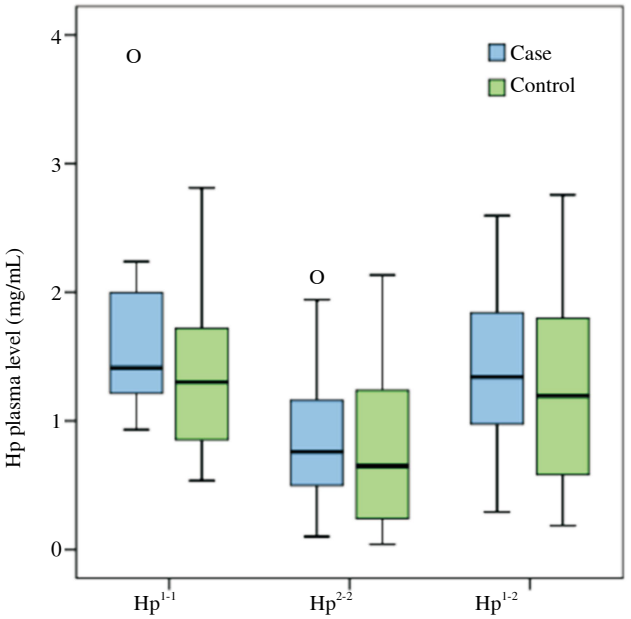

Figure 2. Comparison of plasma haptoglobin concentration by Hp genotypes and study participant category.

4. Discussion

4.1. ABO blood group

The results of this study revealed that ABO blood group proportions in leprosy patients are similar to the equivalent proportions in the population, suggesting that there is no association between ABO blood group and the prevalence of leprosy. This finding agrees with other previous works. For instance, a study in China on 459 Chinese leprosy patients and 15 261 healthy individuals found non-significant difference between leprosy patients and healthy controls[22]. In the same way, studies conducted in Bangladesh and India[23,24] are also consistent with the present study. Furthermore, the analysis of combined data of 27 series from 14 different countries among 11 261 leprosy patients and 390 602 healthy controls brought similar conclusion[25]. However, the analysis of 31 series having 12 299 leprosy patients and 393 023 healthy controls indicated high frequency of "B" and low frequency of "O" in leprosy patients, with lack of significant difference[26].

Therefore, considering the results of present study and many similar conclusions from other studies in different countries, there seems to be no convincing evidence to suggest any association between ABO blood group phenotypes and the occurrence of leprosy.

4.2. Hp polymorphism

Many biological functions of Hp depend on its genotypes due to their CD163 binding affinity difference. Hp^{1-1} has greater hemoglobin (Hb) binding capacity and then Hp^{1-1}-Hb complex has more CD163 binding affinity than Hp^{1-2} or Hp^{2-2}[4]. Thus, Hp^{1-1} is able to bind more Hb on a molar basis than the two phenotypes[27], and clears Hb more effectively than Hp^{2-2}. This phenotype dependent Hb or CD163 binding affinity of Hp influences Th1/Th2 immune response regulation through cytokine production from macrophages exposed to Hp-Hb complex via CD163 dependent mechanism[12,13]. For instance, Hp^{1-1}–Hb complex promotes the secretion of more IL-6 and IL-10 than the Hp^{2-2}–Hb complex[13] and hence, increases Th2 dependent immune response. These distinct Th1/Th2 responses characterize host immune response to *M. leprae* infection along leprosy spectrum[1,14].

The results of this study showed a lower frequency of Hp^{1-1} (14.6%) and higher frequency of Hp^{2-2} (25.6%) in leprosy patients than in healthy controls (23.1% and 19.8%, respectively), with absence of significant association between Hp genotype and occurrence of leprosy. This is in agreement with a study conducted in Angola among 905 leprosy patients and healthy controls which indicated lower frequency of Hp^{1-1} (48.9%) in leprosy patients than in controls (53.9%)[28]. Similar study in South Africa also showed low percentage of Hp^{1-1} in leprosy patients (22.9%) as compared to controls (27.9%), but more frequency of Hp^{2-2} in leprosy patients (30.9%) than in healthy controls (25.8%)[29]. In contrast, a data from India indicated low frequency of Hp^{2-2} in leprosy patients (65%) as compared to controls (71%)[30], and significant association of [Hp0-0] (ahaptoglobinemia; lack of Hp alleles due to deletion[31]) with leprosy prevalence. However, the prevalence of ahaptoglobinemia was not shown in the present study both in leprosy patients and in healthy controls. Thus, the findings of this study and previous works suggest that haptoglobin genotypes may not influence leprosy susceptibility, and the controversial reports may be related to population stratification or genetic heterogeneity biases which could occur in the studied population groups.

4.3. Hp concentration

The results obtained from this study demonstrate that plasma Hp level elevates in leprosy patients and is significantly associated with leprosy spectrum and bacterial loads in patients. These findings are supported by many studies which demonstrated Hp elevation during bacterial infection, inflammation, tissue destruction, and various malignant diseases[3,32] and also consistent with Sritharanand *et al.*[33] who reported high concentration of Hp in untreated LL patients as compared to healthy controls.

Plasma Hp concentration depends on the rate of Hp gene expression and/or Hp release from active neutrophils[3,34]. Besides, Hp gene expression is triggered by anti-inflammatory cytokines particularly IL-6 and IL-1[7,35]. In leprosy, Th2 cells are characterized by their secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 which promote Th2-dominant immune response to *M. leprae* indicating severe poles of leprosy development[17,18]. In contrast, Th1 subtype is characterized by predominant production of pro-inflammatory cytokines like interferon γ (IFN-γ), tumour necrosis factor-beta (TNF-β), and IL-2 which promote Th1-dominant immune response to *M. leprae* indicating the development of a milder poles of leprosy[18,36]. The results of present study together with high IL-6 plasma level and IL-6 mRNA in ENL lesions[37,38] as well as significant rise of IL-4, IL-5, IL-10, and IL-1β cytokines in Multibacillary patients[38] suggest differential Hp expression along leprosy severity. Thus, the increment of Hp level with the severity of leprosy and bacterial loads in patients suggests the possibility of up-regulatory effects of leprosy patient's immune factors on the expression of Hp gene[7].

In conclusion, we report that Hp polymorphism and ABO blood groups may not influence the occurrence of leprosy. However, the elevation of plasma Hp level in leprosy patients and its significant association with bacterial loads as well as considering the strong relationships between leprosy immunology and Hp gene regulatory mechanisms, it may be possible to suggest: 1) the relative increment of plasma Hp concentration with the severity of leprosy is/may be an indication for the increment of Hp gene expression rate with the severity of leprosy; 2) the relative increment of Hp level with leprosy severity is/may be an indication for the shift of leprosy patient's immune response or arises as of more severe form of leprosy.

Conflict of interest statement

We declare that we have no conflict of interests.

Acknowledgment

This study was supported by Armauer Hansson Research Institute. The funder had no roles in study design, data collection and analysis, decision to publication, or preparation of the manuscript.

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