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ORIGINAL ARTICLE

Impact of hepcidin antimicrobial peptide on iron overload in tuberculosis patients

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Abstract

Background: Iron acquisition is essential for the growth of Mycobacterium tuberculosis. Hepcidin is known as an antimicrobial peptide and a component of the innate immune response. Hepcidin inhibits M. tuberculosis growth in vitro. In this study, we decided to identify −582A>G variants of the *HAMP* promoter in patients with tuberculosis (TB) and investigate its effect on serum iron, ferritin, and hepcidin levels. *Methods:* The sample population consisted of 105 patients with TB and 104 healthy individuals. The −582A>G polymorphism was genotyped using a tetra-primers PCR set. Serum levels of hepcidin were determined using an ELISA kit. Statistical analysis was performed using SPSS software. *Results:* The G allele is meaningfully associated with TB disease (95% confidence interval = 2–4.8, $p < 0.000$). Significant differences were seen in the levels of serum iron and hepcidin but not ferritin between the −582A>G polymorphism genotypes. There was significant reverse correlation between hepcidin and iron ($r = -0.849$, $p = 0.006$). *Conclusion:* A high association was found between serum hepcidin levels and the *HAMP* −582A>G variants in patients with TB. These observations indicate a hypothetical role of this polymorphism in iron metabolism. Hepcidin could perhaps be an option for the treatment of TB.

Keywords: *Hepcidin, polymorphism, iron, tuberculosis*

Introduction

Tuberculosis (TB) is one of the most critical infectious diseases in the world. In 2012, it had an incidence about 9.4 million cases and 1.3 million people died from TB. One-third of the worldwide population is generally thought to be latently infected [1], although only 10% of infected individuals develop TB [2]. Iron acquisition is essential for the growth of Mycobacterium tuberculosis (MTB) [3].

Hepcidin is known as an antimicrobial peptide and a component of the innate immune response, and it inhibits MTB growth in vitro [4]. Moreover, this molecule affects a variety of physiological processes and also functions as a signaling mediator in host defense and inflammation [5]. The hepcidin antimicrobial peptide (*HAMP*) gene is mainly expressed in the liver in iron overload states [6]. Hepcidin regulates iron homeostasis by decreasing iron content of blood through inhibition of intestinal iron absorption and macrophage iron release [7–10]. Hepcidin binds ferroportin, a mammalian iron exporter, and causes its internalization and degradation, resulting in the inhibition of iron flow [11]. Ferroportin is expressed highly on duodenal enterocytes and macrophages [12]. When hepcidin levels increase, both dietary iron uptake and recycling iron resulting from decomposition of senescent red cells by macrophages are decreased. MTB in human monocyte-derived macrophages can acquire iron bound to ferritin, transferrin, and citrate [13]; therefore, high hepcidin levels lead to the reduced availability of iron for MTB. Anemia is a common complication of high hepcidin levels due to the reduced iron supply for erythropoiesis in bone marrow [14]. Iron overload and inflammation stimulate hepcidin expression. Hepcidin synthesis is also suppressed by anemia and hypoxia [15,16].

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Review of previous studies indicates that −582A>G variants are associated with hepcidin promoter activity [17–19] and, therefore, may play a role in iron overload. In this study, we decided to identify −582A>G variants of the *HAMP* promoter in TB patients and investigate their effect on serum iron, ferritin, and hepcidin levels.

Material and methods

Sample preparation

The sample population of this study consisted of 105 patients with TB and 104 healthy individuals who had not received regular iron chelation therapy for several months. They were recruited from medical centers of Golestan province in northern Iran. Information on the patients' serum iron and ferritin levels was extracted from medical records. Blood samples were collected, and genomic DNA was extracted from whole blood using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany).

Genotyping of −582A>G variants

The rs10421768 single nucleotide polymorphism located in 582 base pairs (bp) upstream translation initiation codon was genotyped by using a tetra-primers PCR set. Primer design was performed using Gene Runner software (version 3.05; Hastings, USA) and *HAMP* gene sequence information (GenBank accession no. NG_011563) (Table I). PCR was performed using Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Copenhagen, Denmark) with 50–100 ng of DNA and 5 pmol of each primer on a thermal cycler (Bio-Rad, Munich, Germany). PCR conditions included one step initial denaturation (95°C for 10 min), 35 cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 30 s), and a final extension at 72°C for 7 min. Following amplification, PCR products were electrophoresed in a 2% agarose gel (Fermentas, Sankt Leon-Rot, Germany).

Hepcidin ELISA assay

Serum samples were collected from patients by centrifugation of blood samples at 1500 *g* for 10 min. Serum levels of hepcidin were determined using a commercial ELISA kit (DRG, GmbH, Marburg, Germany) according to the manufacturer's protocol. The final solution color was photometrically measured at 450/630 nm wavelength with a microplate reader (Bio-Rad). The 5–95% range in normal healthy adults is 13.3–54.4 ng/ml according to the manufacturer.

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0; Chicago, USA). The frequencies of associations between genotypes and disease were analyzed using chi-squared and Fisher's exact tests. The differences seen in the values of the biochemical indicators among the −582A>G genotypes were tested by the Kruskal–Wallis test. The relationship between variables was estimated by Pearson correlation analysis. A p value < 0.05 was considered significant.

Results

The alleles were determined by product size band; 317 bp for the A allele, 150 bp for G allele, and 440 bp for control, as shown in Figure 1.

The genotype and allele frequencies of the TB patient group and control group are demonstrated in Table II. The wild-type genotype (AA) was observed in 21 (20%) of the patients, whereas 72 (68.6%) were heterozygous (GA) and 12 (11.4%) were homozygous (GG). The control group included 66 (63.5%) individuals with AA, 32 (30.8%) with GA, and 6 (5.7%) with GG. There were highly significant differences regarding −582A>G polymorphism of *HAMP* between TB patients and the control group (Table II). The results indicate that G allele is meaningfully associated with the TB disease (95% confidence interval [95% CI] = 2–4.8, $p < 0.000$). The *HAMP* −582A>G genotypes in TB patients and the control group were in accordance with Hardy–Weinberg equilibrium.

Significant differences were seen in the serum iron and hepcidin levels in −582A>G polymorphism

Table I. Primers used for −582A>G polymorphism determination and sequence analysis.

Description	Primer	Sequence (5′→3′)
−582 A>G	Forward outer	CTTAAGCGATCTGCCTCAG
	Reverse outer	AGGAGTGTCTGGCATGTTG
	Forward inner (G)	GGTCTGACACTGGGAAAACAGCG
	Reverse inner (A)	GTGTGCCCGATCCGCCCGT
Sequence analysis	Forward	TGATATCCCAAAGAAGAGTAGC
	Reverse	CAACTTTCCTGGCAACCTC

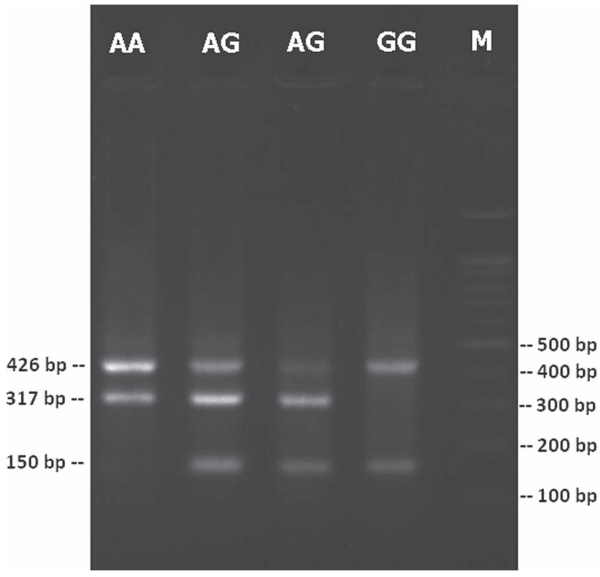

Figure 1. Electrophoresis pattern of PCR for detection of −582A>G polymorphism.

genotypes (Table III). These results indicate that iron levels in GA and GG genotypes have higher values ($p = 0.003$). Unlike iron, hepcidin concentration decreased in GA and GG genotypes ($p = 0.030$). Based on the results in Table IV, there was significant reverse correlation between hepcidin and iron ($r = -0.849$, $p = 0.006$) and no significant correlation between hepcidin and ferritin ($r = 0.151$, $p = 0.341$).

Discussion

MTB as a cause of TB disease requires iron for growth and replication [3]. Hepcidin is an antimicrobial peptide that regulates iron homeostasis [20,21], and better understanding of its role in TB disease may be interesting. With regard to the points mentioned, there are not many studies on the relationship of hepcidin to TB disease development [4,22]. In this work, we genotyped −582A>G polymorphism located in the *HAMP* gene promoter in TB patients and the control group and its relationship with serum iron, ferritin, and hepcidin. In the present work, the correlation of −582A>G variants to TB disease was highly significant (Table II) and no studies have been reported previously. Our results on the association of −582A>G polymorphism to serum iron and hepcidin are in agreement with the study by Andreani et al. [17] and in contrast with work by Bruno et al. that did not find any association [19]. We report a reverse correlation between hepcidin and iron in the way that high hepcidin decreases the iron level (Table IV), as has been reported in previous studies [7,23,24]. The −582A>G variants demonstrated an impact on the level of hepcidin expression and its subsequent increase in serum iron in TB patients. These observations suggest that −582A>G polymorphism may be associated with changes in the *HAMP* promoter function. This *HAMP* promoter substitution, therefore, might represent a risk factor for TB patients by promoting iron overload in them.

Table II. The genotypes and alleles distribution of −582A>G polymorphism in case and control groups.

Characteristic	TB group, *n* (%)	Control group *n* (%)	Odds ratio (95% CI)	*p* value
Genotypes				
AA	21 (20)	66 (63.5)	1	
GA	72 (68.6)	32 (30.8)	7 (3.7–13.5)	<0.0001
GG	12 (11.4)	6 (5.7)	6.1 (2.1–19.8)	0.0009
A/G + G/G	84 (80)	38 (36.5)	6.9 (3.7–12.9)	<0.0001
Alleles				
A	114 (54.3)	164 (78.9)	1	
G	96 (45.7)	44 (21.1)	3.1 (2–4.8)	<0.0001

CI, confidence interval; TB, tuberculosis.

Table III. Levels of serum iron, ferritin, and hepcidin according to *HAMP* −582A>G genotype in patients with tuberculosis (TB).

	−582A>G genotypes			
Characteristic	A/A	A/G + G/G	*p* value	Normal range
n (%)	21 (20)	84 (80)		
Serum iron, mean ± SD (μg/dl)	31.6 ± 17.7	53.5 ± 16.2	0.003	45–160 males; 40–150 females
Serum ferritin, mean ± SD (ng/ml)	31.9 ± 28.7	39.6 ± 35.5	0.045	10–295 males; 25–325 females
Serum hepcidin (ng/ml), geometric mean (95% range)	57.5 (21.9–88.7)	35.2 (17.4–53.1)	0.030	13.3–54.4 adults

Table IV. Relationship between serum hepcidin and serum iron and ferritin in patients with tuberculosis (TB).

Variable	Number	Hepcidin correlation coefficient	*p* value
Serum iron	105	−0.849	0.006
Serum ferritin	105	0.151	0.341

Conclusion

Due to the impact of hepcidin on iron level, differences in the level of hepcidin expression may partially relate to the phenotypic variants in iron hemostasis among TB patients. In the present study, a high association was found between serum hepcidin level and the *HAMP* −582A>G variants in TB patients. These observations indicate a hypothetical role of this polymorphism in iron metabolism. This assay can be useful as part of the diagnostic and prognostic evaluation in TB disease. Hepcidin could perhaps be an option for the treatment of TB.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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