Research Article

Molecular Study of Hepcidin HAMP (-582A/G) Gene Polymorphisms and Measurement of Serum Hepcidin Level among Sudanese Patients with Anemia of Chronic Kidney Disease

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ABSTRACT

Background: Anemia of chronic disease is anemia found in a certain chronic disease state, is typically marked by the disturbance of iron homeostasis or hypoferremia. Chronic renal failure is currently known as Chronic Kidney Disease (CKD) or Chronic Renal Insufficiency (CRI) implies long-standing, progressive, and irreversible renal parenchyma disease resulting in diminished renal function up to 40% to 60%. Often, Chronic Kidney Disease is diagnosed as a result of screening of people known to be at risk of kidney problems, such as those with high blood pressure or diabetes and those with a blood relative with chronic kidney disease. This disease may also be identified when it leads to one of its recognized complications such as cardiovascular disease, anemia, or pericarditis.

Methods: Sysmex KX21 used to CBC and the Cobase411 used to iron profile. Enzyme-Linked Immunoassay (ELISA) was used to determine the level of serum hepcidin.

Results: The results show the significant statistical association observed between the hepcidin level and end-stage kidney disease. When the measured variables compared with different polymorphisms of the HAMP gene, an insignificant relation observed.

Conclusion: This study evaluates for the first time the association between Anemia of Chronic Kidney Disease (ACKD) and Hepcidin (HAMP) genes promoter polymorphisms and show that the hepcidin HAMP (-582 A/G) AA genotype and the allele A are more frequent in patients affected by ACKD, further investigation is needed, our data support the hypothesis and hepcidin HAMP (A/G) are important in the pathophysiology of ACKD.

Keywords: Anemia; Hepcidin HAMP gene polymorphism; Chronic kidney disease patients

Abbreviations: ACKD: Anemia of Chronic Kidney Disease; A: Adenine; CRP: C-Reactive Protein; C: Cytosine; DNA: Deoxyribonucleic Acid; G: Guanine; HAMP: Human Antimicrobial Peptide; HFE: High Iron Fe; IL-6: Interleukin-6; IL-1: Interleukin-1; SPSS: Social Packages Statistical; TNF: Tissue Necrosis Factor; mRNA: Messenger Ribonucleic Acid; NS: Non-Significant

Introduction

Anemia of chronic disease is anemia found in a certain chronic disease state, is typically marked by the disturbance of iron homeostasis or hypoferremia. The kidneys are vital organs that perform a variety of important functions, the most prominent functions are removal of unwanted substances from plasma (both waste and surplus), homeostasis (maintenance of equilibrium) of the body's water, electrolyte and acid-base status, and participation in hormonal regulation. In the clinical laboratory, kidney function tests are used in the assessment of renal disease, water balance, and acid-base disorders and situations of trauma, head injury, surgery, and infectious disease. This chapter focuses on renal anatomy and physiology and the analytic procedures available to diagnose, monitor, and treat kidney dysfunction [1,2].

■ Kidney functions

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Urine formation, fluid and electrolyte balance, regulation of acid-base balance, excretion of the waste products of protein metabolism, excretion of drugs and toxins, secretion of hormones (renin, erythropoietin, 1,25-Dihydroxy vitamin D3, prostaglandins) [2]. Hepcidin, a peptide composed of 25 amino acid, is synthesized by hepatocyte, it inhibits iron release from macrophages, intestinal epithelial cells and placental syncytiotrophoblasts by its interaction with the transmembrane iron exporter ferroportin, accelerating degradation of ferroportin mRNA increased production of hepcidin is induced by inflammatory via interleukin 6 (IL-6), hepcidin synthesis and secretion are controlled by proteins, HFE hemojuvelin, and transferrin receptor.

Materials and Methods

■ Study participants

This study included 100 Sudanese patients diagnosed as anemia of chronic kidney disease, 50 CKD end-stage dialysis-dependent, and 50 CKD non-dialysis who were attended Bahri dialysis center. Besides 50 healthy individuals were recruited from the same center as co-patients and included as healthy control groups. Sysmex kx21 used to RBCs profile, Cobas e-411 used to determine the iron profile and ELISA used to determine the level of hepcidin.

■ Sample preparation and PCR detection of HAMP DNA polymorphisms

The genomic DNA concentration was measured on a nanodrop 1000 spectrophotometer (Thermo-scientific, USA) at 260 nm and 280 nm. The mean concentration of the purified genomic DNA was 20.56 ng/ul, after extraction.

■ HAMP gene promoter region also amplified using published primer set

5 - G T A C T C A T C G G A C T G T A G A T GATTTA GC (forward), 5GTGACAGTCGCTTTTATGGGGCCTGC-3 (reverse). Amplification was done through Polymerase Chain Reaction (PCR) Cycle (95°C, 3 min) × 1, (95°C, 30 s, 55°C, 30 s, 72°C, 60 s) × 37: (72°C, 10 min) × 1, using Dream Tag Green Master Mix Fermenats-Thermo, USA). Restriction digestion of PCR products was done using Fast Digest. As regards the detection of HAMP (-582 A/G) gene polymorphism, Fast Digest HindIII restriction enzyme (Fermentas-Thermo-USA) was used, the digestion products were subjected to 2.5% agarose gel electrophoresis and showed that allele A which did not contain the HindIII restriction enzyme site was digested to 200 bp fragments, whereas allele G yields 190 bp and 90 bp fragments **(Figure 1)**.

■ Statistical analysis

Statistical assessment was carried out with a statistical package for social sciences (SPSS), Genotypes and allele frequencies between groups was analyzed by Chi-Squared test.

Results

The current study show, mean of the RBCs profile (RBCs count, Hb, PCV) (3.353 ± 0.88 cell/l, 10.62 ± 2.4 g/dl, 32.59 ± 6.82%) in patients with ACKD *vs* (4.048 ± 0.47 cell/l, 12.52 ± 1. 57 g/dl, 37.92 ± 4.79%) in control groups p-value (0.00, 0.00, and 0.00) respectively **(Table 1)**. The mean value of the iron profile, S. iron, S. ferritin and TS% (61.353 ± 29, 8 µg/dl, 195.3, 62 ± 19.4 ng/ml, 21.59 ± 12. 82%) in patients with ACKD *vs* (82.048 ± 0. 47 µg/dl, 80.52 ± 1. 57 ng/ml, 28.92 ± 4. 79%) in control groups p-value (0.00, 0.00, and 0.00) **(Table 2)**. Serum hepcidin levels and serum higher in patients with ACKD compared with healthy controls mean (161.55 ± 29.8 ng/ml, 195.322 ± 19.224 ng/l. p-value (0.000) **(Table 3)**. The polymorphisms (SNP) of the hepcidin (HAMP) gene promoter in Sudanese patients with ACKD showed that the hepcidin HAMP (-582 A/G) (SNP) AA genotype 70 (35%), AG 23 (11.5) and GG 7 (3.5%) in 100

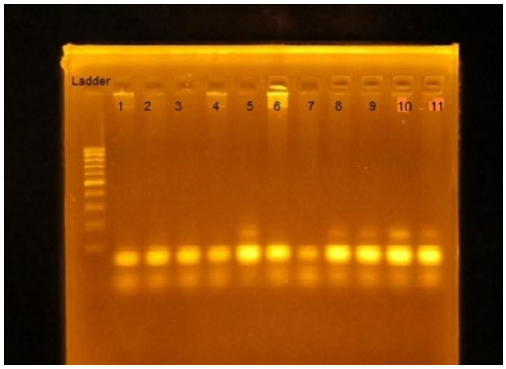

Figure 1: Gel picture of PCR products digest by HindIII restriction enzyme (HAMP). 100 bp DNA Ladder (100 bp-1000 bp). Lane 1, 2, 3, 4, 6, 7: GG homozygote alleles (bands at 90 bp and190 bp). Lane 8, 9, 10, 11: AG heterozygote alleles (bands at 90 bp, 190 bp, and 200 bp). Lane 5: AA homozygote alleles (band at 200 bp).

Table 1: Mean of some red blood cell profile in 200 patients with anemia of chronic kidney disease and 100 control group. S: Significant; NS: Non significant.

RBCs profile	Mean case	Mean control	p-value
RBCs count cell/l	3.353 ± 0.88	4.048 ± 0.47	0.000^{S}
HB g/dl	10.62 ± 2.4	12.52 ± 1.57	0.000^{S}
PCV (%)	32.59 ± 6.82	37.92 ± 4.79	0.000^{S}
MCV fl	93.09 ± 10.12	93.04 ± 4.28	0.974^{NS}
MCH pg	34.95 ± 33.55	30.59 ± 1.42	0.068^{NS}
MCHC g/l	316.34 ± 37.58	368.10 ± 397.37	0.070^{NS}

Table 2: Mean of serum iron profile in 200 patients and 100 control group; S: Significant.

Iron profile	Mean Case	Mean control	p-value
Iron µg/dl	61.55 ± 29.8	82.05 ± 13.4	0.000^{S}
Ferritin ng/ml	195.322 ± 192.24	80.89 ± 60.94	0.000^{S}
TIBC µg/dl	253.97 ± 77.87	260.32 ± 52.49	0.586^{NS}
TS%	21.33 ± 12.72	28.17 ± 4.70	0.000^{S}

Table 3: Mean of Hepcidin levels in 200 patients and 100 control group.

Variables	Mean Case	Mean Control	p-value
Hepcidin ng/ml	61.55 ± 29.8	82.05 ± 13.4	0.000^{S}

Table 4: Frequencies of genotype to the hamp (-582 a/g) gene polymorphisms to 100 patients with ACKD (dialysis dependent) and 100 control group.

	Participants			
Genotype	Case	Control	Total	p-value
AA	70 (35%)	83 (41.5%)	153 (76.5%)	0.076^{NS}
AG	23 (11.5%)	17 (8.5%)	40 (20%)	
GG	7 (3.5%)	0 (0%)	7 (3.5%)	
Total	100 (50%)	100 (50%)	200 (100%)	

Table 5: Comparison of the Hepcidin and all CKD stages (Five stages).

Variables	CKD stages (I)	CKD stages (J)	Mean of difference	p-value
Hepcidin ng/ml	Stages	Stage 1	-8.908	0.007^{S}
		Stage 2	-3.092	0.342^{NS}
		Stage 3	-0.8	0.805^{NS}
		Stage 4	-18.751	0.000^{S}
		Stage 5		

Table 6: Comparison study of the measured variables and different genotypes of HAMP (-582 A/G) polymorphisms.

Variables	Genotypes	Frequencies	p-value
RBCs Count	AA	0.997	0.373
	AG		
	GG		
Hb	AA	1.169	0.315
	AG		
	GG		
PCV	AA	0.848	0.431
	AG		
	GG		
MCV	AA	0.827	0.44
	AG		
	GG		
MCH	AA	0.177	0.838

	AG		
	GG		
MCHC	AA	0.45	0.639
	AG		
	GG		
S. Iron	AA	2.071	0.132
	AG		
	GG		
S. ferritin	AA	0.622	0.539
	AG		
	GG		
TIBC	AA	0.139	0.87
	AG		
	GG		
TS (%)	AA	0.339	0.713
	AG		
	GG		
S. hepcidin	AA	1.237	0.295
	AG		
	GG		

patients dialysis-dependent and AA 83 (41.5%), AG 17 (8.5%) and GG 0 (0%), and the allele A are more frequent in patients affected by ACKD **(Table 4)**. The significant statistical association observed between the hepcidin level and end-stage kidney disease **(Table 5)**. When the measured variables compared with different polymorphisms of the HAMP gene, an insignificant relation observed **(Table 6)**.

Discussion

CKD is at least three to four times more frequent in Africa than in developed countries [3-5], and in Sudan, the prevalence of dialysis patients is presumed to be increasing. Anemia is commonly seen in all stages of kidney disease but much more pronounced in patients with end-stage CKD [6], and is considered an inflammatory state, and increased serum hepcidin levels have been found in patients with ESKD on maintenance hemodialysis. The sequencing of the HAMP gene allowed the identification of a genetic variant with an allele frequency higher than 0, 01, both loci could be considered polymorphic [7]. The variant c-582A-G which results in an allele frequency of 0,762 for c-582A and 0,218 for c-582G. Recent studies have investigated in human inactivating mutations of hepcidin result in a rare form of juvenile hemochromatosis [8], whereas hepcidin overexpression in inflammation causes anemia of chronic diseases [8,9]. We studied the possible association between hepcidin HAMP (-582 A/G) gene polymorphisms and anemia of chronic kidney disease in Sudanese patients. No studies have been performed to test whether the A/G HAMP gene polymorphisms associated with ACKD.

The current study, also focused on measurements of the serum hepcidin levels using the ELISA technique. In this study there is a significant association between CKD and RBCs profile (RBCs count, Hb, PCV) confirming that the patients with CKD are anemic, and the type of anemia found is dimorphic normocytic normochromic and microcytic hypochromic anemia. Hepcidin levels were significantly higher in patients with ACKD compared with healthy controls, comparable results were also reported in other studies [10], who found that the hepcidin levels are higher in patients with ACKD in Indian patients. It has also been indicated that hepcidin levels were approximately two to three-fold higher in patients with ACKD than in the controls [11]. Hepcidin is expected to be elevated in patients with ACKD due to limited hepcidin excretion in urine, tissue iron overload, and inflammation [12]. Also agreed with the results of, who reported that the hepcidin levels are likely to be higher in ACKD patients due to inflammation, and agreed with the results of who found who reported that the hepcidin levels are likely to be higher in Egyptian patients with ACKD [12,13].

Among this study group patients, the decreased levels of serum iron, TIBC, and TS% were

found, However, serum ferritin levels were found to be elevated in this group. Findings consistent to this results agreement with a study on patients with CKD [14] who reported that the serum ferritin levels are likely to be higher in ACKD patients due to inflammation The situation in which the serum iron is low and the serum ferritin is high is frequently seen among ACKD patients, High ferritin levels may be observed in this disease because of functional iron deficiency or reticuloendothelial blockade.

The present study shows the comparison between the study variables (RBCs profile, Iron profile, hepcidin levels) in the all stages of CKD in Sudanese patients, (five stages according to GFR), showed statistically significant differences in the RBCs count, Hb, PCV, S. iron, S. ferritin, TIBC. TS%, hepcidin level in end-stage of CKD (Dialysis dependent), and no statistically significant differences seen in the MCV, MCH, and MCHC, concluding that the severity of CKD can increase the severity of anemia, influencing in the iron status and increase the levels of hepcidin.

The present study show in the first time that the genotype distribution and allele frequency for HAMP (-582 A/G) in Sudanese were compared in all subjects, no significant difference was observed among studied groups. Also, no significant difference was observed in the frequency of HAMP (-582 A/G) alleles among studied groups p-value=0.076. When male and female patients analyzed, a similar distribution of IL-6 G/C and HAMP A/G genotypes and allele frequencies were found p-value 0.747, 0.238 respectively, demonstrating that the HAMP A/G polymorphism is not involved in the pathophysiology of ACKD in both men and women. No significant association between the -582A/G genotype and serum iron, serum ferritin, transferrin saturation, or ferritin levels were found, which might reflect no differences in liver iron concentration. Also, no significant relation was found between HAMP (-582 A/G) variants and the different others studied parameters. These results are in agreement with who found no association between the -582 A/G genotype and serum iron, serum ferritin, and transferrin saturation, and the present study also disagreement with [15], who found that the -582A/G in the human HAMP promoter has no effect on the hepcidin transcription in normal situations but have some effect in pathophysiological situations where more hepcidin was needed. However, from the findings of the present work, the hepcidin HAMP (-582 A/G) genetic variations are unlikely to play an important role in the genetic predisposition to ACKD, conflicting results may be due to various reasons such as demographic features of the subject and different lifestyle, also sample size plays a crucial role. This situation encourages more and more attempts to be made to further assess the associations of these polymorphisms with the disease.

Conclusion

This study evaluates for the first time the association between anemia of Chronic Kidney Disease (ACKD) and Hepcidin (HAMP) genes promoter polymorphisms and show that the hepcidin HAMP (-582A/G) AA genotype and the allele A are more frequent in patients affected by ACKD, further investigation is needed, our data support the hypothesis and hepcidin HAMP (A/G) are important in the pathophysiology of ACKD.

Acknowledgment

By the grace of Almighty Alla and his help I complete this study, all praise to him, my gratitude goes to Dr. Enaam A, Rhman and Dr. Khalid M, Khalid my supervisors whose guides me to complete this work, finally special thanks to patients who were so cooperative and despite their pain.

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