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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 certain chronic disease states, 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.

Sample preparation and PCR detection of HAMP DNA Polymorphisms: Restriction digestion of PCR products was done using Fast Digest. (Figure 1).

Results: Serum hepcidin levels higher in patients with anemia of chronic kidney disease compared with healthy controls mean. The polymorphisms of the hepcidin gene promoter in Sudanese patients with ACKD showed that the hepcidin HAMP AA genotype 70, AG 23, and GG 7 in 100 patients dialysis-dependent and AA 83, AG 17 and GG 0, and the allele A are more frequent in patients affected by ACKD. Significant statistical association observed between the hepcidin level and end-stage kidney disease.

Conclusion: This study evaluates for the first time the association between anemia of chronic kidney disease and hepcidin genes promoter polymorphisms and show that the hepcidin HAMP 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 are important in the pathophysiology of ACKD.

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Introduction

Anemia of chronic disease is anemia found in certain chronic disease states, is typically marked by the disturbance of iron homeostasis or hypoferremia. This condition leads to a shortage of iron for hemoglobin synthesis but the iron storage in bone morrow is left undisturbed. Anemia of chronic disease is the second most prevalent anemia after iron deficiency anemia, it can be triggered by the wide range of inflammatory disorders such as infection, autoimmune disease, chronic diseases aging process and malignancy, also known as anemia of inflammation [1] [2]. Kidney failure is a medical condition in which the kidneys fail to adequately filter waste products from the blood. Studies of hepcidin, an antimicrobial peptide that have a role as a modulator of iron homeostasis has given a new insight for the management of the anemia of chronic disease. 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 on dialysis who were attended Bahri dialysis center. Besides 50 healthy individuals were recruited from the same center as co-patients and included as a healthy control group. Sysmex kx21 used to RBCs profile, Cobas e411 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 concentration of DNA was measured on a nanodrop 1000 spectrophotometer (Thermo-scientific –USA) at 260 and 280nm. The mean concentration of the purified genomic DNA was 20.56ng/ul. After extraction, using published primer set: {5-GTACTCATCGGACTGTAGATGATTTAGC (forward),5GTGACAGTCGCTTTTATGGGGCCTGC-3 (reverse) HAMP gene promoter region also amplified. Restriction digestion of PCR products was done using Fast Digest. As regards the detection of HAMP (-582A/G) gene polymorphism, the Fast Digest HindIII restriction enzyme (Fermentas-Thermo-USA) was used. (Figure 1), 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±0.88cell/l, 10.62±2.4g/dl , 32.59±6.82%) in patients with anemia of chronic kidney disease Vs (4.048±0.47cell/l, 12.52±1.57g/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, serum iron, serum ferritin and transferring saturation percentage (61.353±29,8ug/dl, 195.3.62±19.4ng/ml, 21.59±12.82%) in patients with anemia of chronic

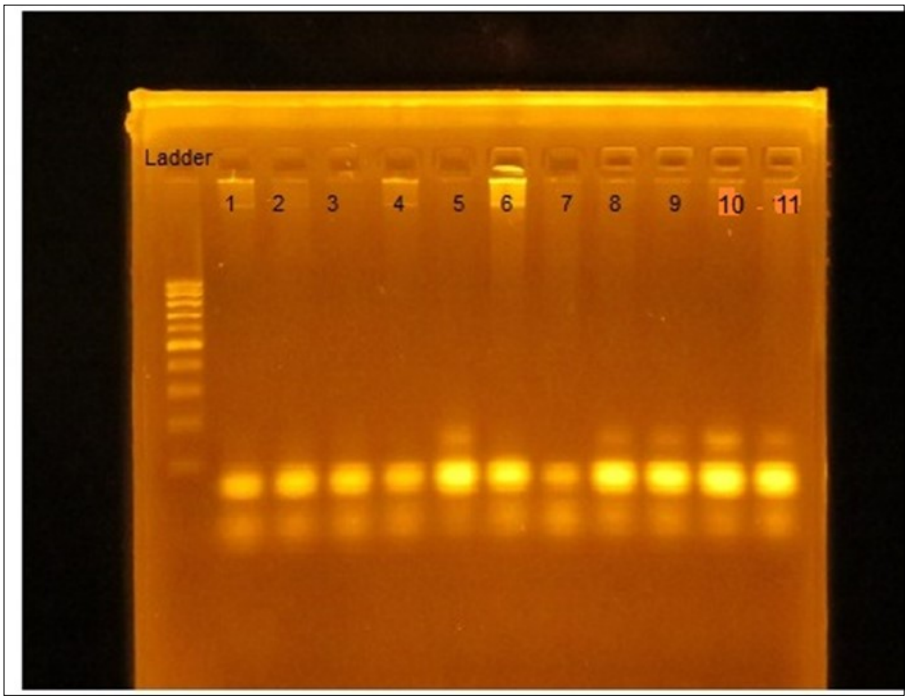

Figure 1. Gel picture of PCR products digest by HindIII restriction enzyme (HAMP). 100 b.p DNA Ladder (100–1000 b.p). Lane 1, 2, 3, 4, 6, 7 : GG homozygote alleles (bands at 90 bp and190 bp). Lane 8, 9, 10, 11: AG heterozygote alleles (bands at 90 b.p, 190 b.p, and 200 b.p). Lane 5: AA homozygote alleles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

Table 1. Mean of Some Red Blood Cell Profile in 100 Patients with Anemia of Chronic Kidney Disease and 100 Control Group

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}

kidney disease Vs (82.048±0.47ug/dl, 80.52±1.57ng/ml, 28.92±4.79%) in control groups P.value (0.00 , 0.00 , and 0.00). (Table 2). Serum hepcidin levels higher in patients with anemia of chronic kidney disease compared with healthy controls, mean (161.55±29.8ng/ml,195.322±19.224ng/l. P.value (0.000) (Table 3). The polymorphisms (SNP) of the hepcidin (HAMP) gene promoter in Sudanese patients with anemia of chronic kidney disease showed that the hepcidin HAMP (-582A/G) (SNP) AA genotype 70 (35%), AG 23(11.5) and GG 7 (3.5%) in 100 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 anemia of chronic kidney disease (Table 4). 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

This current study focuses on the possible association between hepcidin HAMP (-582A/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 CKD. 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 [3], who found that the hepcidin levels were higher in patients with ACKD in Indian patients. It has also been indicated that hepcidin levels were approximately two to three-folded higher in patients with ACKD than in the controls [4]. Hepcidin is expected to be elevated in patients with ACKD due to limited hepcidin excretion in urine, tissue iron overload and inflammation[5]. Also agreed with the results of [6],who reported that the hepcidin levels are likely to be higher in ACKD patients due to inflammation, and agreed with the results of [7], who found who reported that the hepcidin levels are likely to be higher in Egyptian patients with ACKD. 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 in agreement with a study on patients with CKD [8], 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 the 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 (-582A/G) in Sudanese were compared in all subjects, no significant difference was observed among studied groups. Besides, no significant difference was observed in the frequency of HAMP (-582A/G) alleles among studied groups P.value =(0.076). When male and female patients analyzed, a similar distribution of HAMP A/G genotype and allele frequency is found P.value 0.238, 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(-582A/G) variants and the different others studied parameters. These results are in agreement with [9], who found no association between the -582A/G genotype and serum iron, serum ferritin, and transferrin saturation, and the present study also

Table 2. Mean of Serum Iron Profile in 100 Patients and 100 Control Group

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 100 Patients and 100 Control Group

Variables	Mean n 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 (-582A/G) gene Polymorphisms to 100 Patients with ACKD (Dialysis Dependent) and 100 Control Group

Genotype	Participants		Total	P value
	Case	Control		
AA	70(35%)	83 (41.5%)	153(76.5%)	
AG	23(11.5%)	17(8.5%)	40(20%)	
GG	7(3.5%)	0 (0%)	7(3.5%)	**0.076^{NS}**
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	P value
Hepcidin ng/ml	Stages	Stage1	-8.908	0.007^{S}
		Stage2	-3.092	0.342^{NS}
		Stage3	-0.800	0.805^{NS}
		Stage4	-18.751	0.000^{S}
		Stage 5	20.777	0.000^{S}

Table 6. Comparison study of the Measured Variables and Different Genotypes of HAMP (-582A/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	0.440
	AG		
	GG		
MCH	AA	0.177	0.838
	AG		
	GG		
MCHC	AA	0.450	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	0.870
	AG		
	GG		
TS%	AA	0.339	0.713
	AG		
	GG		
S.hepcidin	AA	1.237	0.295
	AG		
	GG		

disagreement with [10], 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 (-582A/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 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.

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

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