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Molecular characterization of Hepcidin (*HAMP* gene exon2) Gene in Selected Iron Deficiency Anemia Patients from Basrah Governorate, Iraq

Ashwaq R. Nazzal[1], Faizah AW Ahmed[2] and Sadiq K. Ali Al-Salait[3]
1,2Department of Biology, College of Education for Pure Sciences, Basrah University, Basrah, Iraq
3Hematopathology Consultant, Center for Hereditary Blood Diseases of Basrah, Iraq

Abstract:

Anemia constitutes one of the most common blood diseases, which can be dangerous and more complex than it appears. This danger comes from the apparent lack of iron which is a vital element in various metabolic and anabolic processes. The *HAMP* gene produces a protein called Hepcidin, which has a very important role in iron metabolism. This study aimed at the molecular detection of *HAMP* gene in selected iron deficiency anemia patients, as well as healthy control, from Basra community in the south of Iraq. Forty five samples were collected from private clinics, which were divided into 33 samples for affected patients and 12 samples represented the control group. A1768 bp fragment containing HAMP exon 2was amplified using Forward: GTGGGACTTGGGGATAAGGC and Reverse: GGGCCTTGCTTTCTTGCTTC . four different polymorphisms were obtained depending on the number of mutations that occurred for the gene compared to what was recorded in the GenBank for the same gene, 3 polymorphisms, studies represented the affected and 1 represented the control, they were all registered in the GenBank under accession numbers LC713271, LC713271, LC713273, (patients' samples), and LC713274, (control samples). The polymorphisms obtained in the current study had a number of different mutations, whether silent or missense, some mutations occurred in more than one polymorphism, while some occurred in one polymorphism. It was noted that some mutations occurred in all polymorphisms of the study. When conducting a BLAST analysis, it was found that the results obtained were closer to each of the genes recorded in America and China, and this can be clearly observed in the analysis of the phylogenetic tree. The results of the analysis of the three-dimensional structure of the expected protein indicated a great match between the polymorphisms of the study . As a result of the occurrence of these mutations, the *HAMP* gene in Iraq has more than one polymorphism, these polymorphisms may be associated with the function of the gene. Therefore, further studies are needed to link this polymorphism to various traits associated with anemia.

Introduction:

Anemia is the most common hematologic disorder, iron deficiency being the leading cause worldwide (Elstrott et al., 2020). Often, anemia is the presenting sign of a more serious underlying condition that, if left untreated, can generate consequent morbidity (Portugal-Nunes et al., 2020).

The *HAMP* gene is encoding protein called Hepcidin which plays a main role to the metabolism of iron via banning the shot of iron from intestinal cells and macrophages (Melis et al., 2008). Several studies indicated that the obstruction of *HAMP* gene role will lead to an increase in iron load (Xu et al., 2021), while in common cases Hepcidin prevent surplus iron absorption in intestinal mucosa and maintains its normal level in the body (Ganz 2011). On the other hand have indicated that the activity of the Hamp gene is related to its different polymorphisms (Ganz 2006). The deficiency of hepcidin causes increase in hemochromatosis, and hepcidin excess may cause iron deficiency, iron-restricted erythropoiesis and anemia due to some mutations in the *HAMP* gene (Kanwar andKowdley 2013). Pandey et al., (2018) also indicated that the occurrence of mutations in the *HAMP* gene could lead to

beneficial effect for some diseases like iron deficiency anemia, while it may be harmful for others particularly those associated with iron overload. So the HAMP genotyping is useful for the decision of treatment and getting rid of iron overload.

The *HAMP* gene encodes hepcidin, an antimicrobial peptide and key iron regulatory hormone. Hepcidin is mainly produced by the liver during conditions of high iron, infection, or inflammation. Hepcidin controls plasma iron levels by binding to the iron exporter ferroportin (SLC40A1; 604653) and inducing its degradation. By decreasing plasma iron levels, hepcidin provides an iron-restricted internal environment inhospitable to microbes, thereby contributing to innate immunity (Malerba et al., 2020). In humans, the HAMP gene is located on the chromosome19, consist of three exons and two introns (Kemnaet al., 2008).

Aim of the study

In the absence of previous similar studies that deals with this gene in Iraq, this study aimed to characterize the HAMP gene in Basrah Governorate, south of Iraq, in selected sample of objects with and without iron deficiency.

Materials and Methods

This study was conducted from May 2021 to June 2022, in Basrah Governorate, southern Iraq (30.536242°N 47.815819°E). The laboratory investigations were carried out in Bayan Lab.

Inclusion criteria:

Patients with microcytic hypochromic anemia, low serum iron, ferritin, and transferrin saturation, and high TIBC and hepcidin, who attended private outpatient clinics.

Exclusion criteria:

Any patient with any type of anemia other than IDA and did not achieve the criteria above.

Control subjects:

Normal volunteers who have normal iron study and serum hepcidin level.

Sampling:

Twenty one samples were collected (13 samples patients and 8 normal control) Three ml of blood was collected from the samples under study in EDTA tubes, then were kept in the freezer until the DNA was extracted.

The Extraction of DNA:

DNA has been extracted according to the method mentioned by Ngole et al., (2022), then the Nano drop has been used to determine the purity and concentration of DNA. It was taken into account that the ratio of 260/280 nm is close to or equal to 1.8.

The design of primer and the amplification of PCR:

The primers were designed according to Yu et al., (2012) for *HAMP* gene exon 2, Forward: 5'GTGGGACTTGGGGATAAGGC3' and Reverse: 5'GGGCCTTGCTTTCTTGCTTC3'. The amplification of PCR was done according Parajes et al., (2010) in gross volume25 µl with 9.5 µl water nuclease free, 2 µl of DNA template (100ng/ml), 0.5 µl (10 µM) of forward primer, 0.5 µl (10 µM) of reverse primer and 12.5 µl of master mix, the PCR conditions were briefed in Table (1). By using 1.5% Ethidium Bromide 0.5 µg/ml-stained agarose gel the PCR product has been detected. A DNA ladder of size 2000 bp was used. Then the PCR product was purified by using Qiagen kit (Germany) QIAquick®.

The analysis of Sequences:

The sequences were analyzed in first BASE laboratory (APICAL) Malaysia. To match the resulting sequences of current study with HAMP gene in Gen Bank the BLAST and Multiple Sequence Alignment have been carried out (Boratyn et al., 2019), depending on the highest match accession numbers have been selected (DQ496109 in UAS and MG679891 in China).

The analysis of phylogenetic tree:

By using Mega-11 (Tamura and Kumar 2021) the phylogenetic tree has been done compared with gene sequences in both USA (DQ469109) and Chia (MG679891).

The 3D structure of protein:

To detect the 3D structure of protein of resulting sequences, the SWISS-MODEL (Waterhouse et al., 2018) has been used.

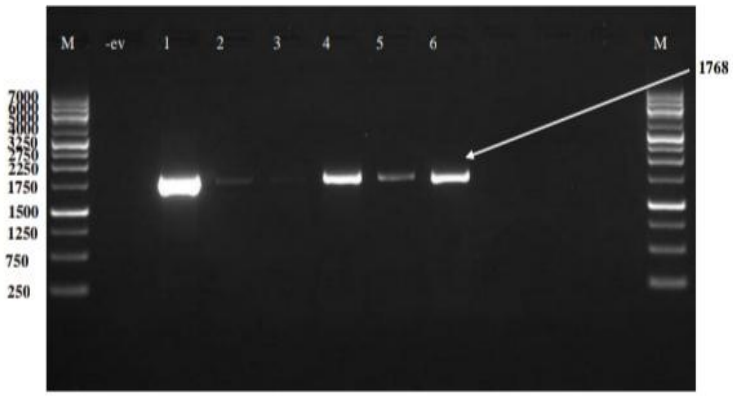
Table1: Cycling protocol and temperature of PCR amplification

Cycle step	Temp (° C)	Time	Number of Cycles
Initial Denaturation	95	5 min	1
Denaturation	94	30 s	30
Annealing	61	30 s	
Extension	72	2 min	
Final Extension	72	10 min	1

Results:

A**nalysis of *HAMP* polymorphisms**

The size of the PCR product was 1768 (Figure 2).Compared with what was recorded in the Gen Bank, four polymorphisms of the *HAMP2* gene were obtained in Basrah Governorate - southern Iraq, they were all registered in the Gen Bank with accession numbersLC7123171 (4 samples), LC7123172 (3 samples), LC7123173 (6 samples),and LC7123174(8 samples) occurred as a result of a number of different mutations compared the patients samples, and LC712374 (8 samples)for control samples (Figure 2), which accession number of USA (DQ496109) and China (MG679891), the highest percentage of match with them. All mutations in the four polymorphisms were summarized in Table 2. A different number of mutations occurred in each polymorphism, some mutations occurred only in patient samples, others occurred in control samples only, and some to a lesser extent occurred in the polymorphisms of patients and the control .On the other hand, when comparing the polymorphisms of the HAMB exon 2 gene resulting from the current study with both the gene registered in America and China, we can notice the presence of mutations that occurred in Iraqn only



Figure(5) : The results of the electrophoresis of a segment of the HAMP gene showed the appearance of bundles of bp nucleotide pair size (1768) between the two sites(1708-1767) using a primer designed for the first time in the current study by primer 3 program .(M:represent DNA Ladder7000bp,80 Volute,45minutes)

	No.	Reference Gene		polymorphism	Position	Mutations	
		DQ496109 USA	MG679891 China			Type	Amino acid
Polymorphism (LC713271)	1	A	A	T	9	Missense	lysine to asparagine
	2	C	C	A	339	Silent	
	3	A	A	T	360	Silent	
	4	G	G	A	588	Silent	
	5	**T**	**G**	**T**	766	Missense	glycine to cysteine
	6	T	T	C	888	Silent	
	7	T	C	T	1105	Missense	Arginine to cysteine
	8	**G**	**A**	**G**	1411	Missense	Arginine to glycine
	9	**G**	**A**	**G**	1566	Silent	
	10	**G**	**A**	**G**	1593	Silent	
	11	**G**	**A**	**G**	1628	Missense	Lysine to Arginine
	12	**G**	**A**	**G**	1657	Missense	Leucine to glutamine
	13	T	T	A	1719	Silent	
	14	C	C	T	1733	Missense	leucine to proline
	15	G	G	T	1760	Missense	Serine to isoleucine
Polymorphism	1	A	A	T	9	Missense	lysine to asparagine

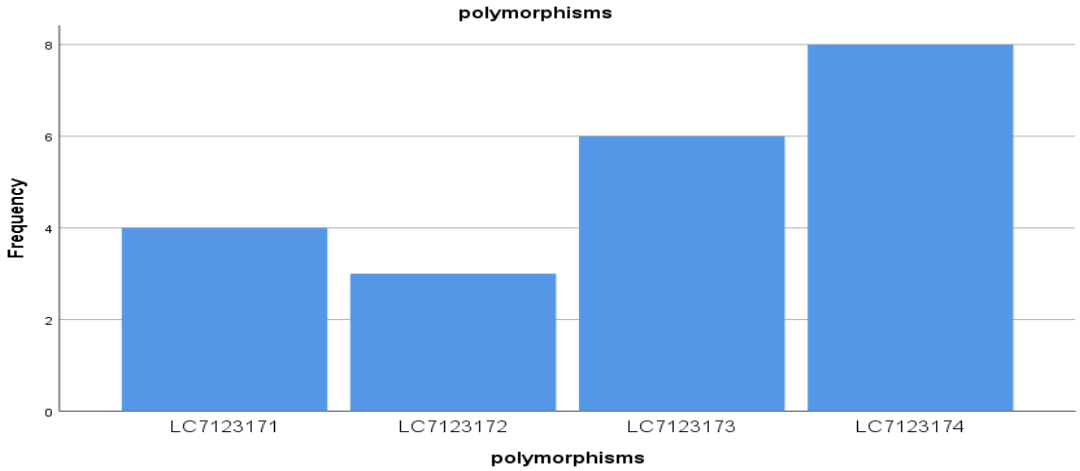
(LC713272)	2	C	C	A	33	Silent	
	3	T	T	C	113	Missense	Valine to Alanine
	4	C	C	G	368	Missense	Arginine to Threonine
	5	T	T	A	369	Missense	Arginine to Threonine
	6	G	G	A	588	Silent	
	7	**T**	**G**	**T**	766	Missense	glycine to cysteine
	8	G	G	C	942	Silent	
	9	T	C	T	1105	Missense	Arginine to cysteine
	10	**G**	**A**	**G**	1411	Missense	Arginine to glycine
	11	**A**	**A**	**G**	1501	Missense	lysine to Glutamate
	12	**G**	**A**	**G**	1566	Silent	
	13	**G**	**A**	**G**	1593	Silent	
	14	**G**	**A**	**G**	1628	Missense	Lysine to Arginine
	15	**G**	**A**	**G**	1657	Missense	Leucine to glutamine
	16	G	G	C	1712	Missense	glycine to Alanine
	17	C	C	T	1733	Missense	Proline to Leucine
	18	G	G	C	1750	Missense	Alanine to Proline
Polymorphism (LC713273)	1	A	A	T	9	Missense	lysine to asparagine
	2	T	T	C	153	Silent	
	3	T	T	G	154	Missense	Serine to Aspartate
	4	C	C	A	155	Missense	Serine to Aspartate
	5	A	A	T	360	Silent	

	6	G	G	A	588	Silent	
	7	**T**	**G**	**T**	766	Missense	glycine to cysteine
	8	C	C	T	1056	Silent	
	9	T	C	T	1105	Missense	Arginine to cysteine
	10	**G**	**A**	**G**	1411	Missense	Arginine to glycine
	11	G	G	T	1520	Missense	Tryptophan to leucine
	12	G	G	A	1521	Missense	Tryptophan to leucine
	13	**G**	**A**	**G**	1566	Silent	
	14	**G**	**A**	**G**	1593	Silent	
	15	**G**	**A**	**G**	1628	Missense	Lysine to Arginine
	16	**G**	**A**	**G**	1657	Missense	Leucine to glutamine
	17	A	A	T	1722	Silent	
	18	C	C	T	1733	Missense	Proline to Leucine
	19	C	C	G	1758	Silent	
	20	T	T	C	1766	Missense	Tryptophan to Threonine
Polymorphism LC713274)	1	A	A	T	9	Missense	lysine to asparagine
	2	C	C	G	368	Missense	Arginine to Threonine
	3	T	T	A	369	Missense	Arginine to Threonine
	4	G	G	T	475	Missense	glycine to Tryptophan
	5	G	G	A	588	Silent	

	6	**T**	**G**	**T**	766	Missense	glycine to cysteine
	7	T	T	A	932	Missense	Leucine to glutamine
	8	T	T	A	933	Missense	Leucine to glutamine
	9	G	G	C	934	Missense	Glutamate to glutamine
	10	T	C	T	1105	Missense	Arginine to cysteine
	11	C	C	T	1167	Silent	
	12	G	G	C	1410	Missense	Aspartate to Glutamate
	13	**G**	**A**	**G**	1411	Missense	Arginine to glycine
	14	**G**	**A**	**G**	1566	Silent	
	15	**G**	**A**	**G**	1593	Silent	
	16	C	C	A	1606	Missense	Leucine to Isoleucine
	17	**G**	**A**	**G**	1628	Missense	Lysine to Arginine
	18	**G**	**A**	**G**	1657	Missense	Leucine to glutamine
	19	G	G	C	1710	Silent	
	20	C	C	T	1733	Missense	Proline to Leucine
	21	T	T	A	1746	Silent	
		C	C	G	1752	Silent	
		A	A	G	1759	Missense	Serine to glycine

Table 2. polymorphism frequency of HAMP2 Gene .

polymorphism	Frequency	Percent	OR(95%Cl)	P. value
LC7123171	4	19.0	0.38124(0.0942-1.5517)	0.1785
LC7123172	3	14.3	0.2500(0.0550-1.1370)	0.0728
LC7123173	6	28.6	0.6000(0.1631-2.2073)	0.4421
LC7123174	8	38.1	control	
Total	21	100.0		



Figure(7)Mutation analysis of HAMP2 Gene

The size of the PCR product was 1768 (Figure 1).Compared with what was recorded in the Gen Bank, four polymorphisms of the HAMP gene were obtained in Basrah Governorate - southern Iraq, they were all registered in the Gen Bank with accession numbersLC123171 (4 samples), LC123172 (3 samples), LC123173 (6 samples), and LC123174 (8) for control samples (Figure 2), which accession number of USA (DQ496109) and china (MG679891), the highest percentage of match with them. All mutations in the four polymorphisms were summarized in Table 1) (

1-The genetic change appeard in the nucleotide site 9 , c.A>T in which the amino acid Lysine was replaced by asparagines in all samples, including the control samples, where missnes were present in all of them, while the mutation was silent at sites 360 and 1722.

2- Shifting of the cytocine base to adenine C>A led to a change in the amino acid serine to asparagines at acid sites 155,1606, while the mutation was silent at site 33.

3- T>C Thymine change to Cytocine at sites 113,1766 were missnese and it was silent in 888 and 153.

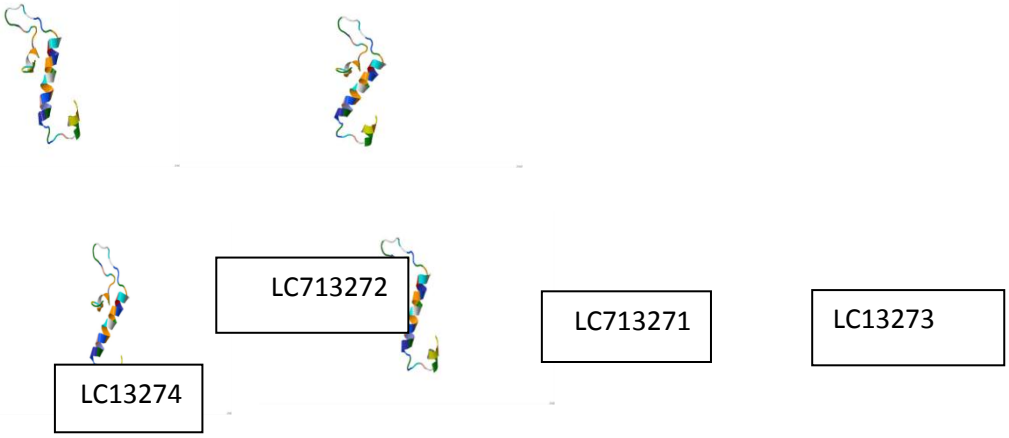
4- T>A missnes on sites 369, 932 , while was silent on1719 and 1746

5- C>T Cytocin to Thymin on sites 1760, 1733,1056 and was silent on 1767

6- G>T were all the missnes at sites 1760,152, 475

7-G>A at position 588

8- A>G at position 1759 and 1501



Figure(8):3 structur protein of hamp2 mutations

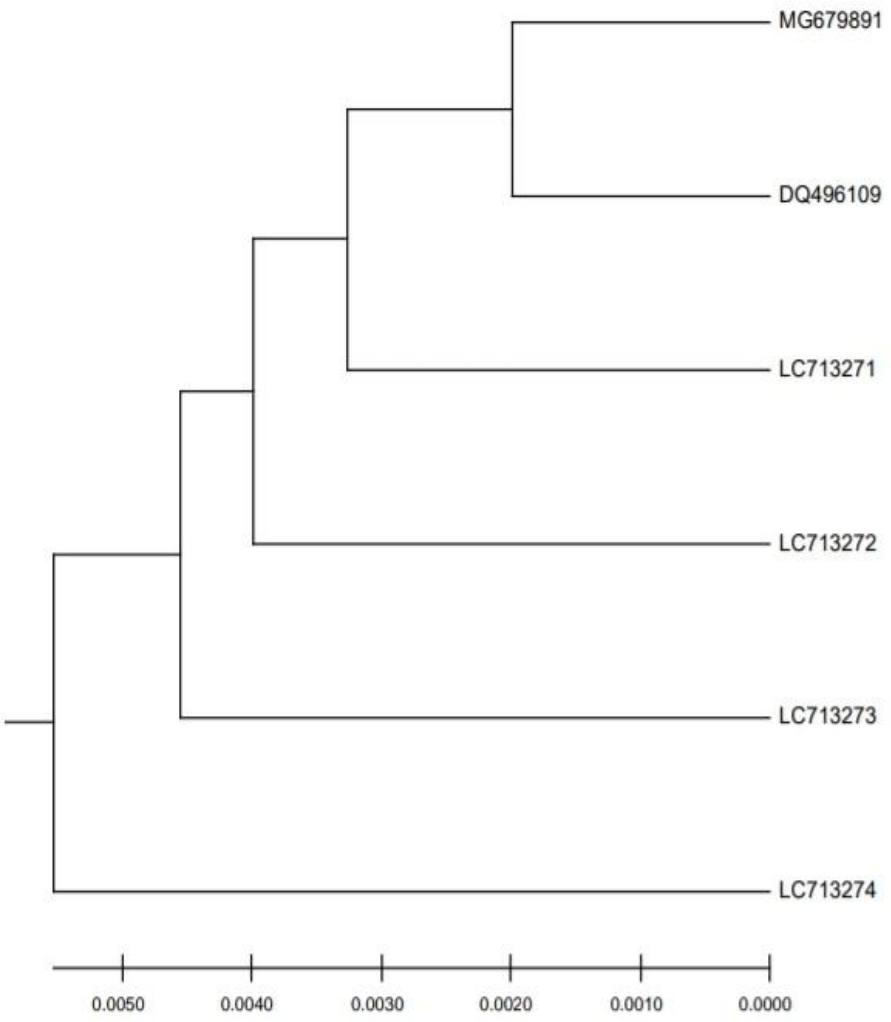
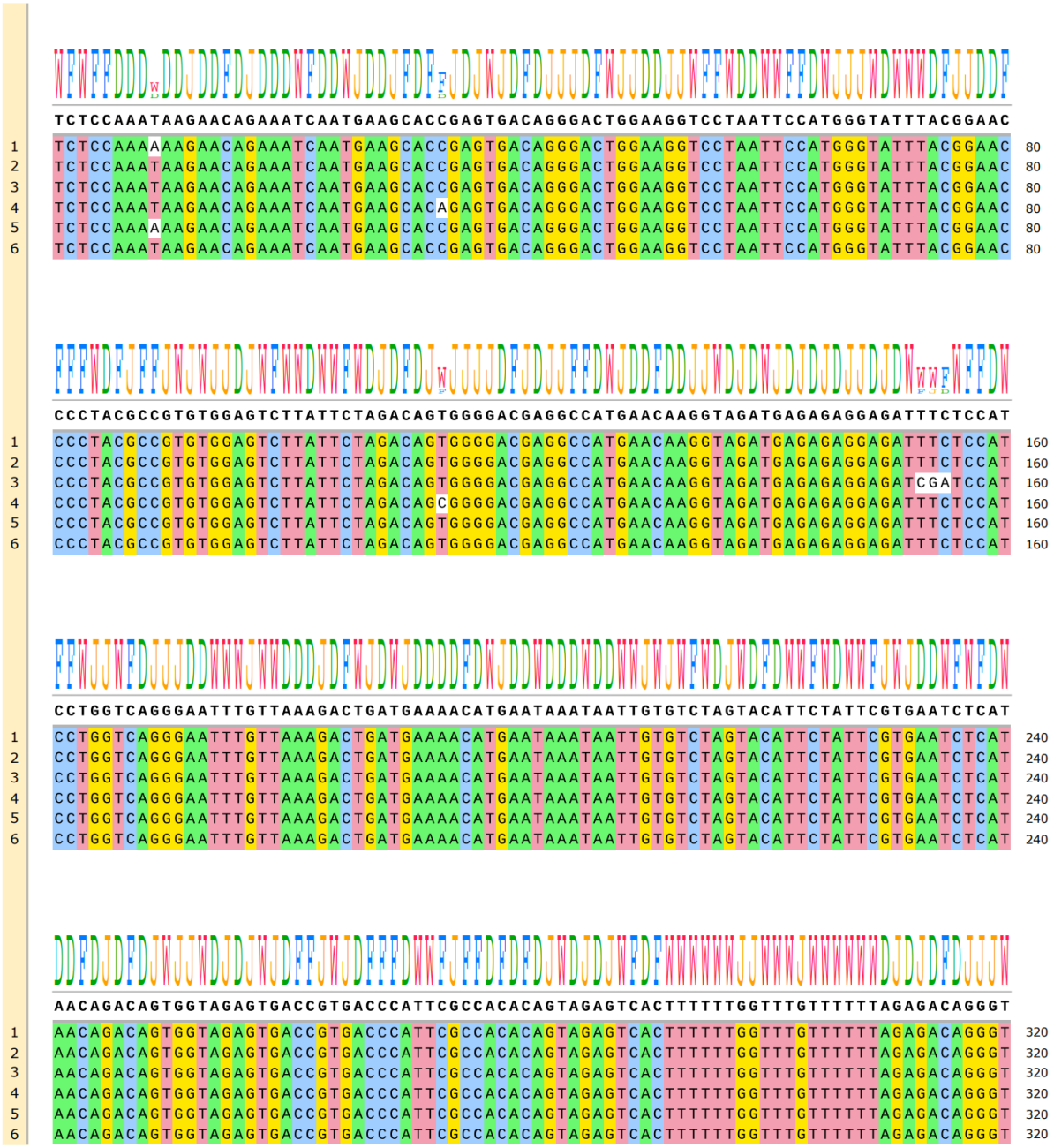
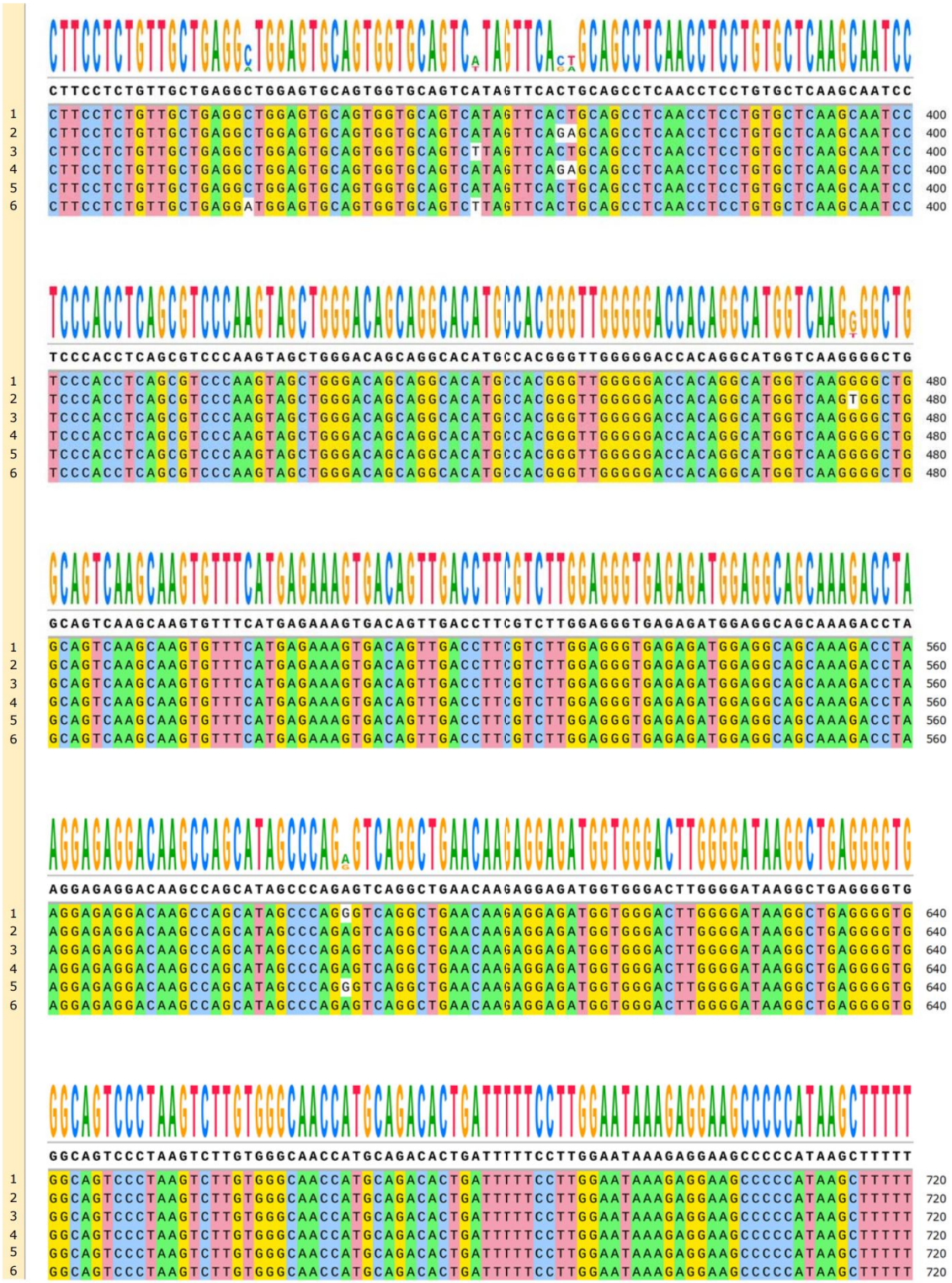


Figure 8: The Phylogenetic tree of HAMP2 gene in Iraq as well as USA and China (LC713271, LC713272, LC713273,and LC713274, : HAMP 2 gene in Iraq), (DQ496109): HAMP 2 gene in USA). (MG679891: HAMP gene in China).

Aligned using an external algorithm

Consensus
1. MG679891
2. LC713274
3. LC713273
4. LC713272
5. DQ496109
6. LC713271

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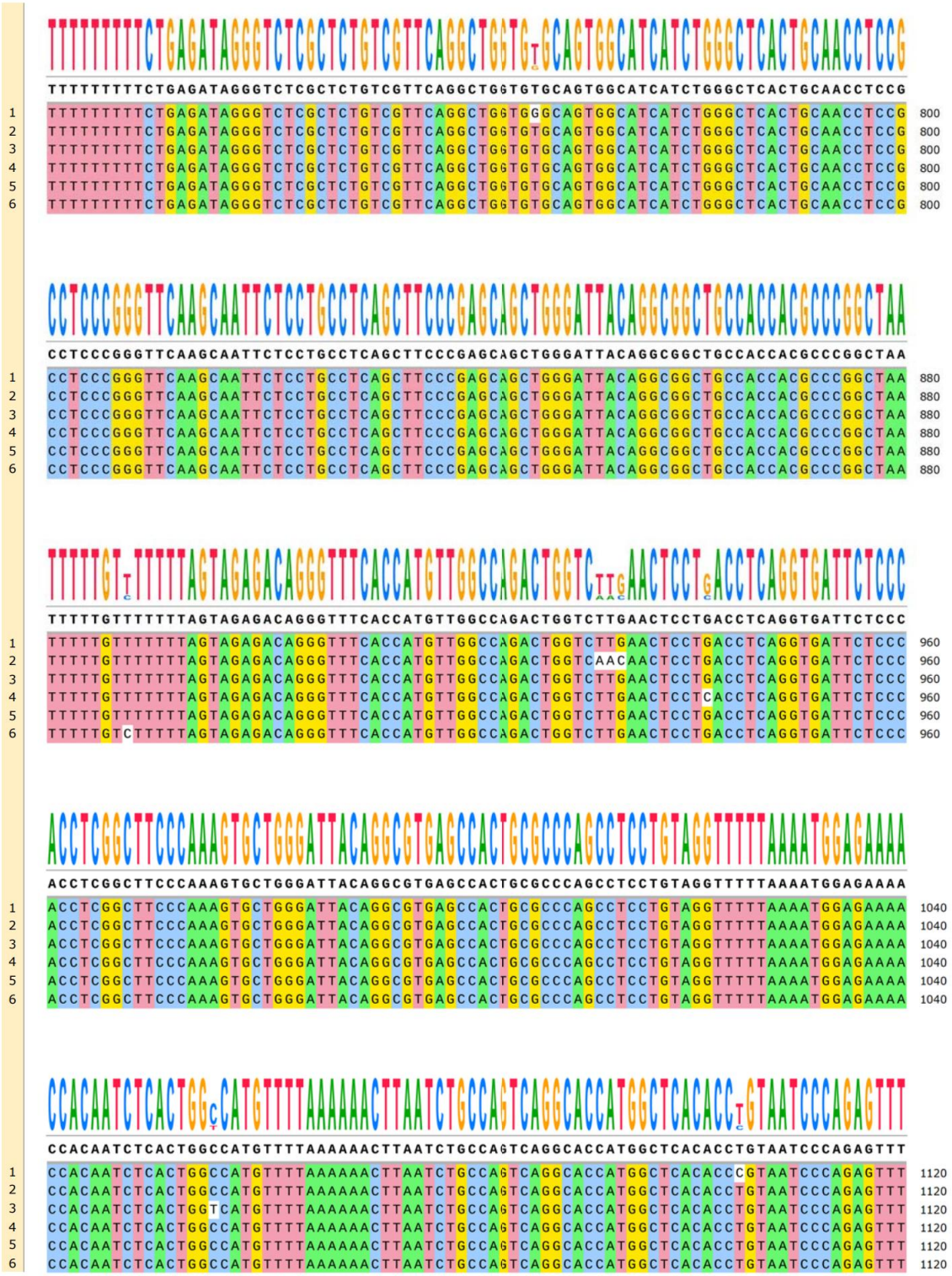
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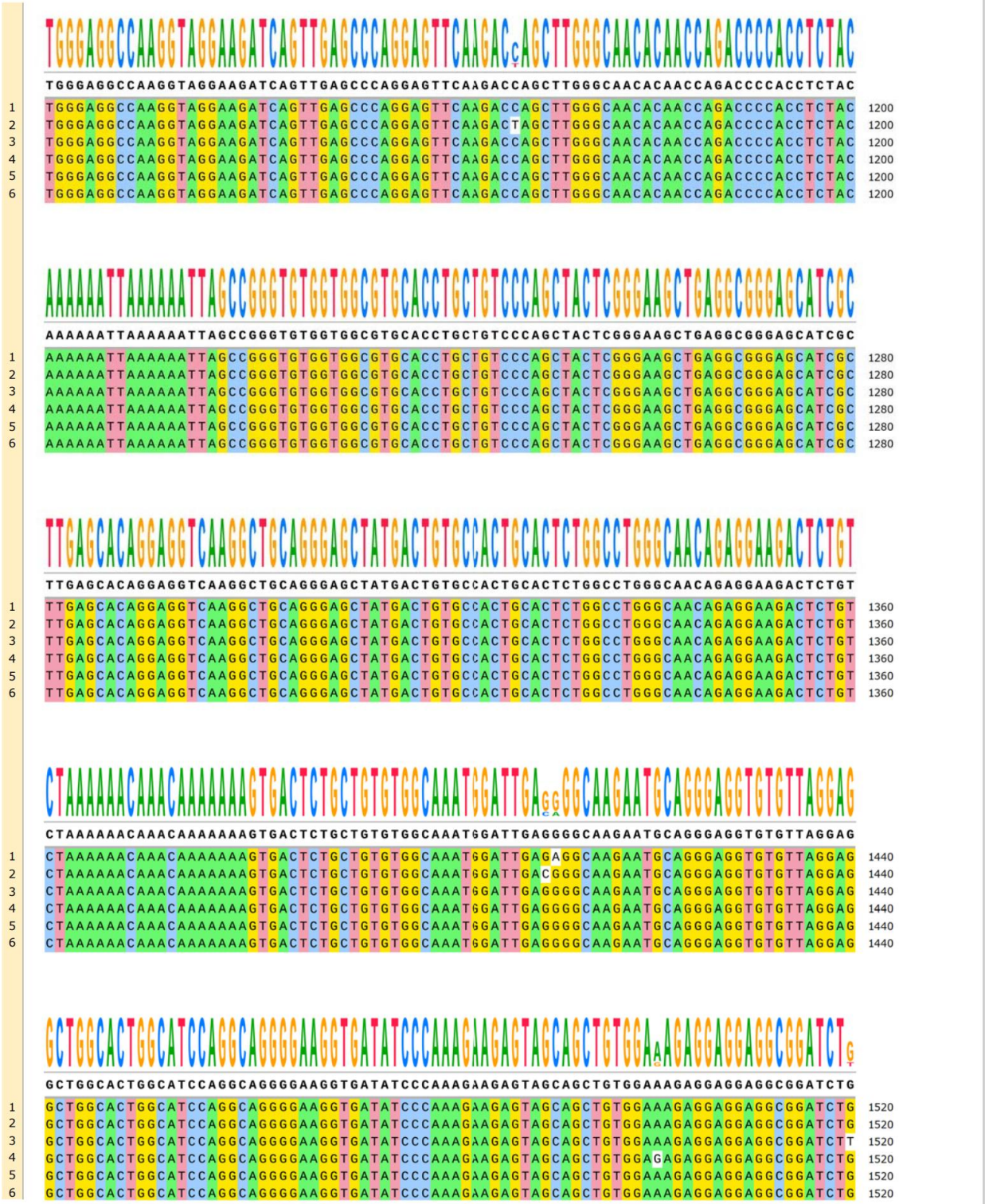
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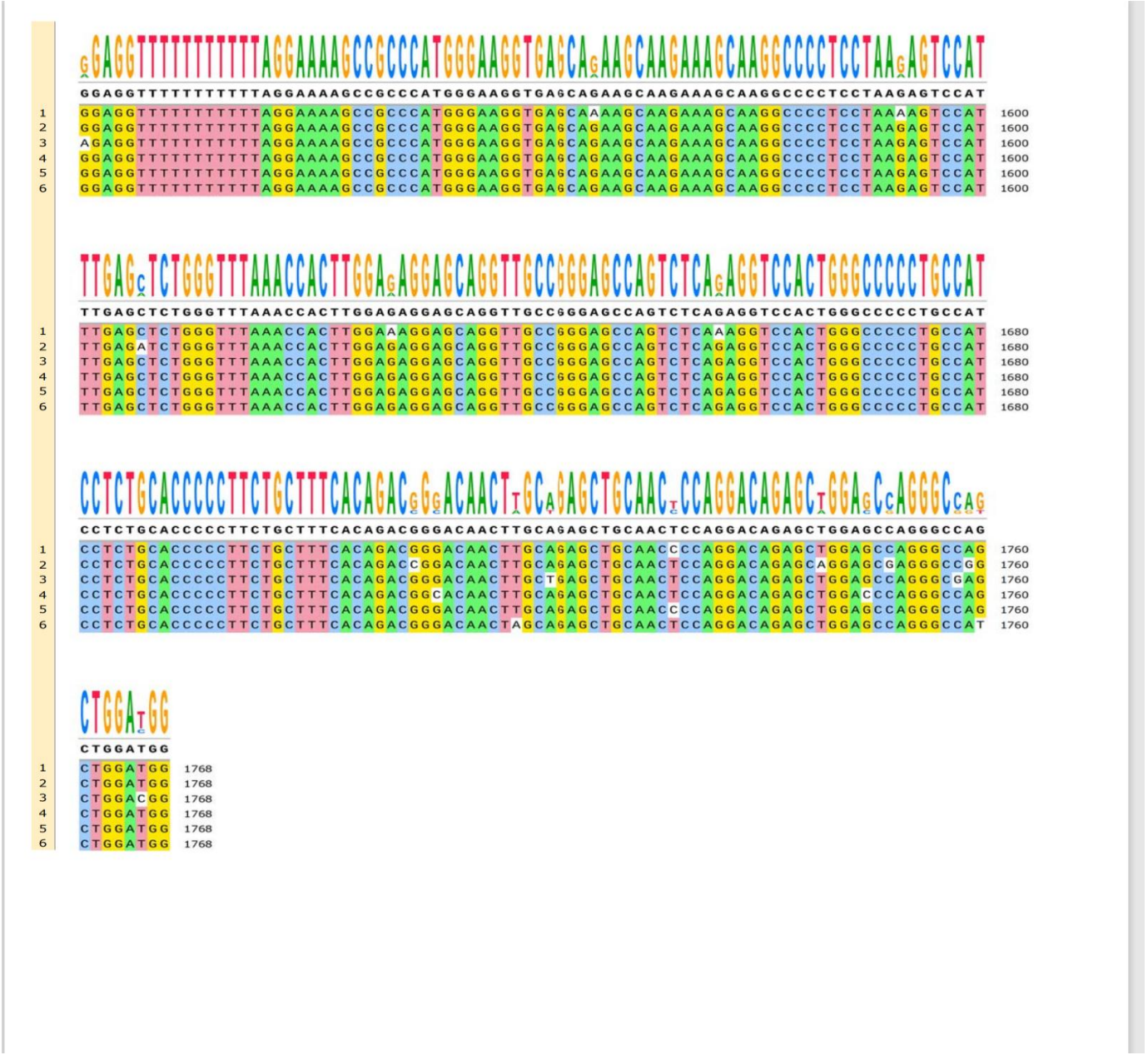

Figure 5: The Multiple Sequence Alignment for each of the USA and China polymorphisms, in addition to the polymorphisms of the current study. 2,3,4,, ,and 6: Iraqi polymorphisms, LC713271, LC713272, LC713273, and LC713274 respectively. 5: USA polymorphism, DQ496109. 1: China polymorphism, MG679891.

Discussion:

The results of current study agreed with McGregor (2009) about the size of PCR product, (Note that the size of the PCR product in the current study was adopted in order to give a clearer perception of the gene, given that this study is, up to our best knowledge, the first that deals with the study of gene sequences of HAMP in Iraq. The results of this study are in agreement with previous studies on the possibility of polymorphisms of the HAMP gene (McLachlanet al., 2017; Abdelrahman et al., 2020;Jallow et al., 2020). The frequency of some mutations in more than one polymorphism may be due to the fact that the samples were collected from people of similar or common ancestry (Meyerson et al., 2020). The occurrence of silent mutations can have an effect as it

can cause a change in the structure of the protein and thus directly affect its work as well as it can affect the folding of the protein, which can lead to instability of the protein function and affect the interaction with other biological molecules (Peyton 2021). Silent mutations affect related proteins by altering the transcription process and the accuracy and efficiency of mRNA splicing (Komar 2007).On the other hand, the effects of missense mutations come from being caused by a change in amino acids, thus, it can negatively affect the function of the protein or even positively in other cases (Khayat et al., 2021), in other words, these mutations may be the main cause of diseases (Zhang et al., 2012), or, on the contrary, it may contribute to disease resistance(Sun et al., 2021). This difference in effect may be due to the difference in the characteristics of the same amino acids, if there is often a difference (even if a little) between the amino acid before the mutation and the amino acid resulting from the occurrence of the mutation (Mohajeri and Ashrafi 2011), hence the change in the properties of the protein itself. they were the most closely related to the global polymorphisms that were studied which could lead us to the fact that the infected samples in Iraq that were diagnosed in the current study had a number of mutations that clearly affected the gene, which made them genetically far from what exists in the world and thus this could affect negatively or positively on the activity of the protein that It is produced by the gene, and this requires us to study more broadly, linking the polymorphisms obtained in the study with the different characteristics that are directly or indirectly affected by the work of the gene (Zhang et al., 2019).

The clear convergence in the expected protein 3D structure was in all the polymorphisms obtained in the study, this could lead us to study gene sequences even in some cases where the sample is not infected to get a complete picture of the gene's work (Hicks et al., 2019) .

HAMP2 gene polymorphisms

A comparison with the Gen Bank records revealed four distinct HAMP2 gene polymorphisms in the Basrah Governorate of southern Iraq. These polymorphisms have been registered in the Gen Bank under accession numbers LC7123171 (4 samples), LC7123172 (3 samples), LC7123173 (6 samples), and LC7123174 (8 samples) for patient samples, and LC712374 (8 samples) for control samples (Figure 2). The highest percentage of similarity was found with accession numbers from the USA (DQ496109) and China (MG679891).

Different mutations were observed in each polymorphism, with some mutations exclusive to patient samples, others to control samples, and a few occurring in both patient and control sample polymorphisms to a lesser extent. Furthermore, a comparison of the HAMB exon 2 gene polymorphisms from the present study with those registered in America and China revealed mutations unique to Iraq.

Mutations in the four polymorphisms of hamp2 gene overview:

At nucleotide site 9, a missense mutation (c.A>T) was observed in all samples resulting in the replacement of lysine with asparagine. Missense mutations were also present in all samples at amino acid sites 155 and 1606 due to the shift of cytosine base to adenine, while site 33 showed a silent mutation. Thymine to cytosine substitution at sites 113 and 1766 resulted in missense mutations, while sites 888 and 153 were silent. At sites 369 and 932, a missense mutation (T>A) was observed, while sites 1719 and 1746 had silent mutations. Cytosine to thymine substitution at sites 1733, 1056, and 1760 resulted in missense mutations, while site 1767 showed a silent mutation. All missense mutations at sites 152, 475, and 1760 were due to guanine to thymine substitutions, while the missense mutation at site 588 was caused by guanine to adenine substitution. Positions 1759 and 1501 showed missense mutations due to adenine to guanine substitution.

LC713271 polymorphism

Focusing on the LC713271 polymorphism, Table 4 provides a comprehensive comparison of various HAMP2 gene mutations located at distinct positions against reference genes from China (MG679891) and the USA (DQ496109).

Within this polymorphism, a total of 15 mutations were detected, of which 8 were missense mutations that cause alterations in the protein's amino acid sequence. These missense mutations comprise lysine to asparagine, glycine to cysteine, arginine to cysteine, arginine to glycine, lysine to arginine, leucine to glutamine, leucine to proline, and serine to isoleucine. Besides the missense mutations, 7 silent mutations were identified, which do not lead to changes in the protein's amino acid sequence. These silent mutations are located at positions 339, 360, 588, 888, 1566, 1593, and 1719.

The comprehensive analysis of these mutations in the LC713271 polymorphism, as well as their comparison to the reference genes from China and the USA, contribute to the understanding of HAMP2 gene variability and its potential implications in the studied population.

LC713272 polymorphism

When discussing the findings related to the LC713272 polymorphism, the table reveals a range of HAMP2 gene mutations at various positions, compared to their respective reference genes.

A total of 18 mutations were detected within the LC713272 polymorphism, with 12 being missense mutations that result in changes to the amino acid sequence of the protein. The observed missense mutations encompass the following alterations: conversion of lysine to asparagine, valine to alanine, arginine to threonine (identified twice, at positions 368 and 369), glycine to cysteine, arginine to cysteine, arginine to glycine, lysine to glutamate, lysine to arginine, leucine to glutamine, glycine to alanine, proline to leucine, and alanine to proline.

In addition to missense mutations, the analysis revealed 6 silent mutations that do not lead to modifications in the amino acid sequence of the protein. These silent mutations were found at positions 33, 588, 942, 1566, and 1593.

The LC713273 polymorphism

In the discussion of the HAMP2 gene mutations within the LC713273 polymorphism, the table revealed a variety of mutations at distinct positions, compared to their respective reference genes. A total of 20 mutations were identified, including 10 missense mutations, resulting in changes to the amino acid sequence of the protein. These missense mutations included lysine to asparagine, serine to aspartate (identified twice at positions 154 and 155), glycine to cysteine, arginine to cysteine, arginine to glycine, tryptophan to leucine (found twice at positions 1520 and 1521), lysine to arginine, leucine to glutamine, proline to leucine, and tryptophan to threonine. Additionally, six silent mutations were found at positions 153, 360, 588, 1056, 1566, and 1593, and two more silent mutations were detected at positions 1722 and 1758.

The LC713274 polymorphism

The results of the mutations within the LC713274 polymorphism of the HAMP2 gene are presented in the table. A total of 21 mutations were identified, includpolymorphism.nse mutations, leading to changes in the amino acid sequence of the protein. These missense mutations included lysine to asparagine, arginine to threonine at positions 368 and 369, glycine to tryptophan, glycine to cysteine, leucine to glutamine at positions 932 and 933, glutamate to glutamine, arginine to cysteine, aspartate to glutamate, arginine to glycine, leucine to isoleucine, lysine to arginine, and serine to glycine. Nine silent mutations were also found at positions 588, 1167, 1566, 1593, 1710, 1733, 1746, 1752, and 1759. By comparing these mutations to reference genes and examining them within the LC713274

polymorphism, further understanding of the variability of the HAMP2 gene in the studied population can be obtained.

The frequency of the HAMP2 gene polymorphisms

Regarding the frequency of the HAMP2 gene polymorphisms in the studied, the distribution pf the four polymorphisms was as follows: LC7123171 (19%), LC7123172 (14.3%), LC7123173 (28.6%), and LC7123174 (38.1%). The total frequency of all the polymorphisms was 100%, with a total of 21 cases identified. The p-value for the analysis was 0.1785, with an odds ratio (OR) of 0.38124 (95% confidence interval: 0.0942-1.5517) for LC7123171, p-value of 0.0728, with an OR of 0.2500 (95% CI: 0.0550-1.1370) for LC7123172, and p-value of 0.4421, with an OR of 0.6000 (95% CI: 0.1631-2.2073) for LC7123173. The LC7123174 polymorphism was used as a control with a frequency of 38.1%. These findings provide insight into the prevalence of the HAMP2 gene polymorphisms in the studied population and their potential association with diseases or health outcomes in this population.

Conclusion and recommendations:

The results of this study clearly show the presence of more than one polymorphism of the *HAMP2* gene in the sample tested. Those polymorphisms may be considered regarding gene function; therefore, it is necessary to conduct more detailed studies on the relationship of the polymorphisms of the gene to the different physiological characteristics, as well as pathological conditions related to iron metabolism, especially since the studies that dealt with this gene in Iraq are very few.

This study adds valuable insight to the field of genetics and may have implications for the development of targeted interventions for IDA patients in Iraq and beyond. Nonetheless, further research is needed to investigate the complex genetic interactions involved in IDA and to develop more effective prevention and treatment strategies.

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