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Haptoglobin Gene Polymorphism among Type 2 Diabetic Mellitus Patients in Gaza Strip-Palestine

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Abstract: Diabetes mellitus (DM) and its complications are a major health problem worldwide, and long-term vascular complications are the leading cause of the morbidity and mortality. Genetic factors are probably involved in the development of this microvascular complication. Haptoglobin (Hp) is a genetically polymorphic glycoprotein that forms stable complexes with plasma-free hemoglobin (Hb) providing protection against heme-induced oxidative stress and kidney damage. There are three common haptoglobin genotypes, 1-1, 1-2 and 2-2, which are determined by 2 alleles, HP1 and HP2, located on chromosome 16q22.

Objectives: Investigating the association between the HP gene polymorphism and type 2 diabetes mellitus (T2DM) patients and compered it with healthy control in Gaza strip-Palestine.

Methodology: A retrospective case-control study was conducted by using 129 subject, 67 T2DM and 62 healthy individual as a control group. EDTA whole blood sample was collected from all participants for DNA extraction. Polymerase Chain Reaction (PCR) was used to detect Hp gene polymorphism. Also, all individuals were requested to fill the questionnaire.

Results and Conclusions: The most frequent genotype in both T2DM and control group was Hp2/Hp2, where it was 47.8% of T2DM patients and 54.8% of the control. Therefore the result was not statistically significant where ($p > 0.05$).

Keywords: Haptoglobin (HP) gene, Haptoglobin genotypes, Type2 diabetes mellitus (T2DM), Polymerase Chain Reaction (PCR).

Introduction

Haptoglobin (Hp) is a Hemoglobin-binding serum protein. It is an acute phase protein synthesized mainly in the liver and found at levels of 30–300 mg/dL in normal human serum [1]. During the acute phase reaction and the response to injury Hp serum levels are increased up to 3- to 8-fold [2]. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1 induce the hepatic synthesis of Hp. Besides to the hepatic synthesis of Hp, it is expressed in specific non-hepatic cells such as "lung cells and adipocytes" and after inflammation its levels are increased similar to that observed in hepatocytes [3]. Additionally, Hp is highly expressed in arteries after sustained flow changes induced by shear stress and nitric oxide, which influence IL-6 expression. Arterial Hp is believed to play a role in cell migration and arterial restructuring [4]. The best-known function of *Hp* is to bind free Hb released into the plasma to form a stable Hp-Hb complex. The two proteins form a complex with extremely high affinity (K_D estimated to be greater than 10^{-15}M) [5]. *HP* plays an essential role in capturing free Hb, thus preventing its deposition in the glomeruli and proximal tubule cells of the kidney. Moreover, *HP* functions as an antioxidant through its ability to bind Hb and thereby preventing oxidative tissue damage mediated by free Hb [6] [7]. *Hp* forms a soluble complex

with Hb, an oxygen-binding tetrameric (α2β2) protein containing a protoporphyrin ring complexed with Fe^{2+} (heme). The binding of *Hp* with Hb is characterized by a very high affinity ($>10^{10}$ mol^{-1}) and stability. The β globin chain of human Hb contains two specific binding sites for *HP*, at amino acid residues β11-25 and β131-146, whereas the Hb-α globin chain has one *Hp*-binding region, comprising residues α 121-l27. Hb α-β dimers bind stoichiometrically to *Hp* α-β subunits. The Hp-Hb complex enhances the peroxidase activity of Hb . The binding of myoglobin to *Hp* is relatively weak and quantitatively is much less important [8].

Haptoglobin gene is located on the long arm of chromosome 16 (16q22.3) consists of 5 exons encoding the 1 allele, or 7 exons encoding the 2 allele. *Hp* phenotype refers to the distinct set of polymeric Hp molecules produced from the two classes of *Hp* alleles. The polymeric nature of the Hp molecule (dimer, linear or cyclic polymer) is dependent on the *Hp* alleles because the protein product of the 1 allele is monovalent, combining with only one other Hp monomer, while the protein product of the 2 allele is bivalent, combining with two other Hp monomers [9]. A signature pattern of polymeric species is, therefore, obtained from individuals who are homozygous for the 1 allele (*Hp 1-1*), homozygous for the 2 allele (*Hp 2-2*), or are heterozygous at the haptoglobin locus (*Hp 2-1*). This unique pattern of polymeric species first demonstrated over 45 years ago by Smithies [10]. HP is synthesized as a single chain, which is cleaved to an amino-terminal α-chain and a carboxyl-terminal β-chain, linked by disulfide bonds [11]. The β-chain is identical in both *HP* alleles, while the α-chains differ. The α-chain of the *Hp1* allele binds one β-chain and one other α-chain, both by disulfide bonds. Therefore, subjects with the *Hp1-1* genotype produce a single $(\alpha\beta)_2$ homodimer with a molecular weight of 86 KDa. As the cysteine forming the intermolecular disulfide bond between the α-chains is duplicated in the two allele, human is homozygotes for the two allele (*Hp2-2*) have multimeric cyclic haptoglobin molecules with a molecular weight of 170–900 KDa. Individuals with the *Hp2-1* genotype demonstrate a variety of linear homodimers and multimers with a molecular weight of 86–300 KDa [11]. Hp protein phenotypes are easily distinguished by no denaturing gel electrophoresis of Hb-enriched serum. The Hp-Hb complex in the gel is identified by virtue of its peroxidase activity, and each *Hp* type gives rise to a signature-banding pattern. The *Hp* type can also be determined from DNA by PCR [12] and there exists complete correspondence between the *Hp* type assigned by the two methods.

Diabetes and its complications are a major health problem worldwide, and long-term vascular complications are the leading cause of morbidity and mortality. According to the Palestinian Ministry of Health, it is the fourth leading cause of death among Palestinians after cardiovascular disease, cerebrovascular disease and cancer. The percentage of deaths due to diabetes and its complications is 8.9% of the total number of deaths among Palestinians, where T1DM comprises about 4,7% of the total diabetic patients in Palestine while T2DM patients comprises about 95.3%. Of the total number of Palestinian refugees living in the Gaza Strip in 2015, 16,889 were males and 23,118 were women with diabetes, according to UNRWA. According to the Palestinian Health Information Center, the rate of diabetes among Palestinians in 2016 rose to 192.8 new cases per 100,000 population [13]. In this study, as the first study in Palestine, which specializes in finding the genotypes of the haptoglobin gene in T2DM patients among the population of the Gaza Strip.

Materials and Methods

1. Ethical considerations

Permission to conduct the study was obtained from Medical Sciences Department graduate committee University Collage of Science and Technology (UCST) and the Ministry of Health in the Gaza Strip.

2. Sampling and sample size

EDTA blood samples were collected from 129 subject, included 67 T2DM patients and 62 healthy individual as a control group. Convenience sampling technique was used to select the samples and all the subjects were Palestinian and above 25 years old.

3. Primers for Hp gene polymorphism

Hp gene primers sequences were used as it reported by Koch, et al. [12]

Table (1): Primer sequences for the detection of HP genotypes

Gene	Primer	Oligonucleotide Sequence (5'-3')	Amplicon (bp)
Hp1	*Hp1 F* *Hp1 R*	GAGGGGAGCTTGCCTTTCCATTG GAGATTTTTGAGCCCTGGCTGGT	1757
Hp2	*Hp2 F* *Hp2 R*	CCTGCCTCGTATTAACTGCACCAT CCGAGTGCTCCACATAGCCATGT	349

4. PCR amplification of *Hp* gene

To determine the *Hp* polymorphism of case and the control subjects, specific primer sequences listed in Table (1) were used to amplify the fragments containing *Hp* gene by using PCR. Two µl (~150ng) of prepared DNA template was added to 10 µl master mix and 2 µl of each primer in 0.2 ml thin walled micro-centrifuge PCR tube. The micro-centrifuge tubes were centrifuged and then placed in a thermal cycler and PCR amplification was done according to the program described below in Table (2) and (3). Upon completion of PCR, the products were analyzed by electrophoresis on 2% ethidium bromide stained agarose gel.

Type of Cycle	Temperature (ºC)	Time	No. of Cycles
Initial Denaturation	**95**	**2 min**	**1**
Denaturation	**95**	**1 min.**	**35**
Annealing	**70**	**2 min.**	
Extension	**72**	**2 min.**	
Final Extension	**72**	**7 min.**	
Cooling	**4**	**Hold**	**Hold**

5. Amplification protocol and temperature cycling program

Two amplification protocols were used in this study, one for Hp1 and the other for Hp1.

Table (2): Amplification protocol for HP1

Type of Cycle	Temperature (ºC)	Time	No. of Cycles
Initial Denaturation	**94**	**5 min.**	**1**
Denaturation	**94**	**30 sec.**	**30**
Annealing	**58**	**45 sec.**	
Extension	**72**	**45 sec.**	
Final Extension	**72**	**7 min.**	
Cooling	**4**	**Hold**	**Hold**

Table (3) Amplification protocol for HP2

6. Expected PCR results

The amplicon (PCR product) generated from *HP* gene should yield a 1757 bp with *Hp1*genotype and 349 bp with *Hp2* genotype. A negative control (nuclease free water instead of the DNA template) was included in each reaction. The size of the amplicon was estimated by comparing it with a DNA molecular size marker (250 kbp ladder DNA to *Hp1* and 100bp for *Hp2*) run on the same gel.

7. Data Analysis

The data was analyzed by using statistical package for Social Sciences (SPSS) version (20.0); Independent Samples t-test, Chi square test and Odd's Ratio (OR) were used to compare the case and control groups in this study. A *p*-value < 0.05 was considered to be statistically significant

Results

1. General characteristics of study population

The study was conducted on 160 individuals who were divided into two groups. The case groups included 67 T2DM patient (mean age 56.03±10.370) and and healthy control group included 62 subject (mean age 44.08±12.539). 76% of study population is non-smoker, while 24% is smoker. 64.3% of the participants were males, while about 35.7% were females. 78.3% of subjects non-hypertensive while 21.7% of them were hypertensive. All healthy control population was non-hypertensive and non-diabetic.

2. Clinical characteristics of study population

The clinical characteristics of the study population were as it shown in Table (4) and (5). The study showed that there was a statistical significant in diabetes with diabetes history and HTN between case and control group (*p*-value < 0.05) while there was no statistical significant with obesity and HTN history (*p*-value > 0.05) as shown in Table (5).

Table (4) clinical characteristics of control, T2DM and

			T2DM
		N	%
Nephropathy	**Yes**	6	9.0
	No	61	91.0
Neuropathy	**Yes**	16	23.9
	No	51	76.1
Heart problems	**Yes**	11	16.2
	No	56	83.6
Retinopathy	**Yes**	18	26.9
	No	49	73.1

T2DN subjects in the studied population.

***All the control group subjects was healthy of this problems**

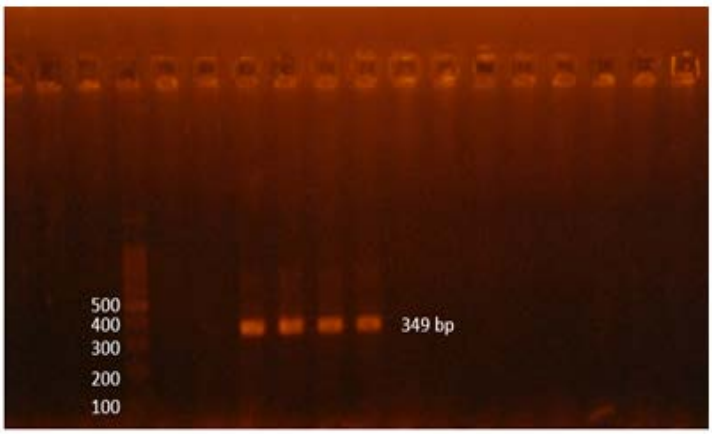
Table (5) Other Clinical characteristics of study population

*According to WHO the individual with BMI more than 30 were been considered as obese and those with a BMI less than 30 were grouped into non-obese population.

		Control		T2DM		*p*-value
		N	**%**	**N**	%	
Family history for diabetes	**Yes**	9	36	48	71.6	0.001*
	No	16	64.0	19	28.4	
HTN	**Yes**	1	1.6	27	40.3	0.000*
	No	61	98.4	40	59.7	
Family history for HTN	**Yes**	11	44.0	33	39.3	0.451
	No	14	56.0	34	50.7	
Obesity	**Yes**	8	32.0	25	37.3	0.636
	No	17	68.0	42	62.7	

3. PCR Results

The quality of the isolated DNA were been tested by running the DNA samples on ethidium bromide stained 1% agarose gel. After the PCR reactions, the amplicons which were obtained, were subjected to 1 % agarose gel electrophoresis for *Hp1* and 3% agarose gel electrophoresis for *Hp2* with ethidium bromide and the bands visualized under UV light. With the help of DNA ladder, *Hp1* allele and *Hp2* allele were identified at 1757 and 349 bp fragment respectively.



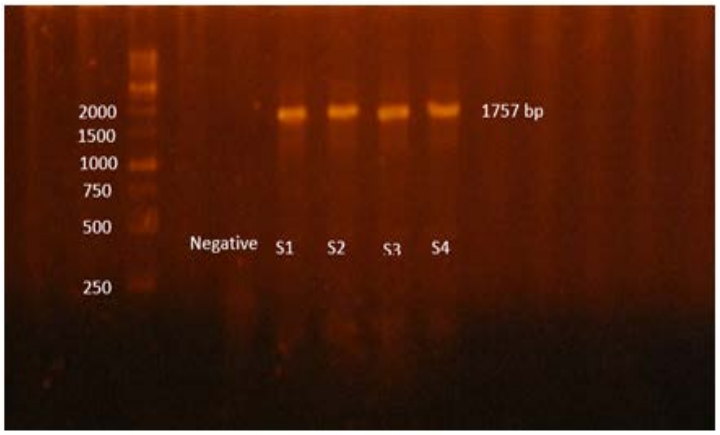


Figure (1): PCR amplification product for *Hp1* Figure (2): PCR amplification product for HP2

3.1 Distribution of all subjects according to Hp Genotypes

As it shown in Figure (3), Hp2-2 genotype is the highest frequency among the studied population.

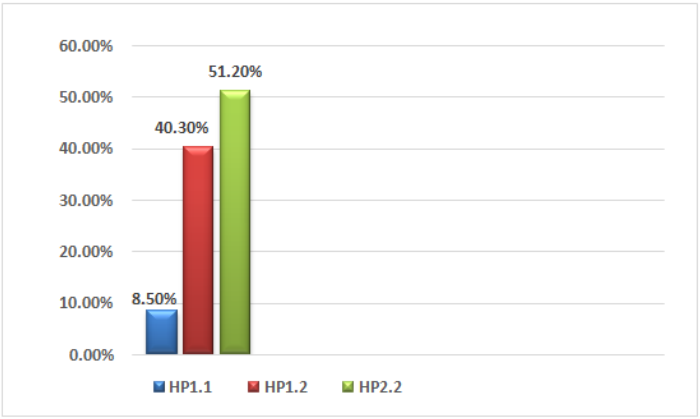


Figure (3): Distribution of all subjects according to *HP* Genotypes

3.2 Distribution of *Hp* genotypes in subjects:

In this study, we found the distribution of the subjects according to *Hp* genotype was {*Hp1/Hp1* 4.8% (3/62), *Hp2/Hp1* 40.3% (25/ 62) and *Hp2/Hp2* 54.8% (34/ 62) among healthy control and in T2DM it was 11.9% (8/67), 40.3% (27/67), 47.8% (32/67). This result was not statistically significant ($p > 0.05$) as shown in Table (6). *Hp2/Hp2* genotype was the most frequent in the female, where this result was statistically significant ($p < 0.05$).

Table (6) Distribution of *Hp* genotype in subjects.

	Hp Genotypes			Total
	Hp1/Hp1	*Hp1/Hp2*	*Hp2/Hp2*	
	N (%)	N (%)	N (%)	N (%)
Control	3 (4.8)	25 (40.3)	34 (54.8)	62 (100.0)
T2DM	8 (11.9)	27 (40.3)	32 (47.8)	67 (100.0)
Total	11 (8.5)	52 (40.3)	66 (51.2)	129 (100.0)
***p*-value**		**0.330**		
Male	6 (7.2)	41 (49.4)	36 (43.4)	83 (100.0)
Female	5 (10.9)	11 (23.9)	30 (65.2)	46 (100.0)
***p*-value**		0.018*		

3.2 The frequency of *HP* gene allele:

Our study found that the *Hp2* allele is the highest frequency a among the studied groups where its frequency was 75% in control and 67.9% T2DM group, while the frequency of *Hp1* allele was 25% and 32.1% respectively) as shown in Table (7).

Table (7): The frequency of *HP* gene allele

Allele	Control N %	T2DM N%
Hp1	31 (25)	43 (32.1)
Hp2	93 (75)	91 (67.9)
Total	124	134

3.3 Relationship between *Hp* genotypes with HTN and obesity state in T2DM patients.

The results showed that there was no statistical significant association between *Hp* genotypes with HTN and obesity state ($p > 0.05$) . The *Hp2/Hp2* genotypes and thus *Hp2* allele were the most frequency among the hypertensive and obese population as shown in Table (8).

Table (8): Distribution of *Hp* genotype in hypertensive and obese population of T2DM patients.

		Hp Genotypes			*p*-value
		Hp1/Hp1	*Hp1/Hp2*	*Hp2/Hp2*	
		N (%)	N (%)	N (%)	
HTN	**Yes**	4 (14.8)	9 (33.3)	14 (51.9)	0.602
	No	4 (10.0)	18 (45.0)	18 (45.0)	
Obesity	**Yes**	4 (16.0)	9 (36.0)	12 (48.0)	0.693
	No	4 (9.5)	18 (42.9)	20 (47.6)	

Table (9): The frequency of Hp gene alleles

		Alleles		*Total*
		Hp1	*Hp2*	
		N (%)	N (%)	
HTN	**Yes**	17 (31.5)	37 (68.5)	54 (100.0)
	No	26 (32.5)	54 (67.5)	80 (100.0)
Obesity	**Yes**	17 (34.0)	33 (66.0)	50 (100.0)
	No	26 (31.0)	58 (69.0)	84 (100.0)

3.4 Relationship between Hp genotypes and nephropathy, kidney diseases, neuropathy and retinopathy in T2DM patients.

Our results found that there was no statistical significant association between *Hp* genotypes with nephropathy, heart problems, neuropathy and retinopathy ($p > 0.05$). The *Hp2/Hp2* genotypes was the most frequency genotype among all the studied cases as shown Table in (8).

Table (10): Relationship between *Hp* genotypes with nephropathy, kidney diseases, neuropathy and retinopathy in T2DM patients.

		Hp Genotypes				*p*-value
		Hp1/Hp1	*Hp1/Hp2*	*Hp2/Hp2*		
		N (%)	N (%)	N (%)	Total	
Nephropathy	**Yes**	0 (0.0)	2 (7.4)	4 (66.7)	6 (100%)	0.507
	No	8 (13.1)	25 (41.0)	28 (45.9)	61 (100%)	
Heart problems	**Yes**	0 (0.0)	5 (45.5)	6 (54.5)	11 (100%)	0.410
	No	8 (14.3)	22 (39.3)	26 (46.4)	56 (100%)	
Neuropathy	**Yes**	0 (0.0)	8 (50.0)	8 (50.0)	16 (100%)	0.221
	No	8 (15.7)	19 (37.3)	24 (47.1)	51 (100%)	
Retinopathy	**Yes**	1 (5.6)	8 (44.4)	9 (50.0)	18 (100%)	0.615
	No	7 (14.3)	19 (38.8)	23 (46.9)	49 (100%)	

		Alleles		Total
		Hp1 N (%)	Hp2 N (%)	
Nephropathy	Yes	2 (25.0)	6 (75.0)	8 (100.0)
	No	41 (33.6)	81 (66.4)	122 (100.0)
Heart problems	Yes	5 (22.7)	17 (77.3)	22 (100.0)
	No	38 (33.9)	74 (66.1)	112 (100.0)
Neuropathy	Yes	8 (25.0)	24 (75.0)	32 (100.0)
	No	35 (34.3)	67 (65.7)	102 (100.0)
Retinopathy	Yes	10 (27.8)	26 (72.2)	36 (100.0)
	No	33 (33.7)	65 (66.3)	98 (100.0)

Table (11): The frequency of *Hp* gene alleles

3.5 OR and 95% CI in the genotype and alleles distribution of HP *gene* polymorphism between the groups

A comparison was done to test the relationship between *Hp* genotypes and T2DM. The OR, CI and *p*-value were calculated for each associate, as shown in Table (12).

Table (12): OR and 95% CI in the *Hp* genotype distribution between the case and control group.

Hp genotype	T2DM / Control Group No.	Odds Ratio (95% CI)	p-value
Hp2/Hp2	32 / 34	0.753 (0.377-1.505)	0.422
Hp1/Hp1+Hp1\Hp2	35 / 28		
Hp1/Hp2	27 / 25	1.001 (0.495-2.024)	0.998
Hp2/Hp2+Hp1\Hp1	40 / 37		
Hp1/Hp1	8 / 3	0.375 (0.095-1.483)	0.149
Hp2/Hp2+Hp1\Hp2	59 / 59		
Hp2/Hp2	32/34	0.871 (0.421-1.803)	0.711
Hp1\Hp2	27/25		

Discussion

According to our results obtained from PCR the *HP* Genotypes frequencies were 8.5% for *Hp1/Hp1*, 40.3% for *Hp1/Hp2* and 51.2% for *Hp2/Hp2* genotype in Gaza Strip. The frequency of *Hp1/Hp2* allele polymorphism of the *Hp* gene in the current study compared to different ethnic groups in different studies. *Hp2/Hp2* genotype was the highest frequency in Gaza Strip (the present study), Iran [14], India [15 , 16] and china [17], while the *Hp1/Hp2* genotype was the highest frequency in France [18] and Japan [19] as shown in Table (13).

Country	Hp1/Hp1(%)	Hp1/Hp2(%)	Hp2/Hp2(%)	Reference
Iran	8.2	40.8	51.3	Bowman JE et al.,1964
India	2.5	13.3	84.2	Padma T et al.,1988
China	9.4	35.2	55.4	Zhao H et al.,1993
France	15.3	49.7	35.0	Van Sande M et al.,1963
Japan	7.4	37.2	35.4	Shindo S et al.,1990
Palestine	6.9	36.3	56.9	The Present Study

Table (13) Distribution of *Hp* gene in different human population

To our knowledge, this is the first report showing the association of *Hp* gene polymorphism with diabetes in Palestine, where we did not find a statistical association between the *Hp* genotypes and the incidence of diabetes. *Hp2* allele was the most frequent among the studied groups where its frequency was 75% in control and 67.9% T2DM group, while the frequency of *Hp1* allele was 25% and 32.1% respectively). This results in concordance with other studies done in America [20], Brazil [21] and Japan [22]. In addition, the results showed that *Hp2/Hp2* genotype was the most frequent in the female, where this result was statistically significant ($p < 0.05$).

However, our results showed that there was no statistical significant association between *Hp* genotypes with HTN ($p > 0.05$). *Hp* polymorphism has been suggested as a candidate genetic marker in essential hypertension [23], and *Hp1* allele frequency is high among patients with essential hypertension [24]. On the other hand, the *Hp2/Hp2* genotype has been associated with increased risk for essential hypertension [25], accumulation of atherosclerotic lesions in essential hypertension, besides showing higher therapeutic needs and more refractory hypertension [24]. Also, in another study done in Brazil authors indicated that Brazilian individuals carrying *Hp2/Hp2*

genotype can present a lower risk of developing hypertension than *Hp1/Hp1* subjects [26]. In another study done in Egypt [27] which found that the frequency of *Hp1/Hp1* was greater in diabetics with normo-albuminuria whereas *Hp2/Hp2* frequency in diabetics with macro-albuminuria.

Recommendation

As this study is the first to investigate the relation between *Hp* polymorphism and type 2 diabetes mellitus in Gaza Strip, we recommended for additional studies by using a larger sample size with the studying of the direct complications of the DM such as dialysis, HTN and CVD. In addition, the authors recommended T2DM patients for at least semiannual control and check for their general health status to avoid the complication of diabetes. Finally we advices the adults who have a family history for T2DM to check up annually to eliminate progress of T2DM since they have a high susceptibility for the disease.

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