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Genetic studyof haptoglobinpolymorphisms in cardiovascular and type 2 diabetes Mellituspatients

Abdulhussien M, Aljebory[1] ,Noor ali[2]
[1]College of Pharmacy, Babylon University,Iraq.
[2]College of Science ,Al-Muthanna University, Iraq

Abstract

This study was conducted to detect of relationship between haptoglobinand cardiovascular disease in diabetes patient byPCR assay for the first timein Al-Muthanna province – Iraq, during the period from October-2013 up to March–2014. The study diagnosed two types of haptoglobinby PCR assay (HP1 1757 and HP2 349).

Key word: Molecular, Haptoglobin, Diabetes Mellitus, Cardiovascular disease.
Correspondingauthor: E.mail:nooralsalami@ymail.com.

Introduction

Diabetes mellitus (DM) is a chronic disease that affects the lives of about 285 million people around the world, with type 2 D.M. making up about 90% of the cases of death (1). It is a metabolic disease characterized by hyperglycemia, either because the body does not produce enough Insulin, or because cells dos not respond to the insulin that is produced (2).

Robbins and contran, 2004(3); has been suggested that diabetic patients carrying the Hp2-2 genotype have increased risk of developing CVD. However, this does not seem to be a consensual observation, and would appear to depend on the population and aspects analyzed. As far as we know, there are few studies investigating the influence of Hp polymorphism on the occurrence of ischemic cardiovascular events, systemic arterial hypertension (SAH), refractory hypertension (RH), obesity and dyslipidemia, and none of these include Brazilian patients with DM.

Haptoglobin (from Greek, haptein: to bind) is a plasma protein that owes its name to its hemoglobin-binding properties. These properties have led to its description as an antioxidant protein because binding to free hemoglobin inhibits hemoglobin-induced oxidative tissue damage (4). Haptoglobin is mainly synthesized by hepatocytes in the liver (5,6), its levels increasing during inflammation or infection (7) Thus, the involvement of haptoglobin in states of oxidation and inflammation has generated interest in its potential association with vascular disease, especially under conditions of increased glycemic levels.

In humans, two alleles for haptoglobin have been described that give rise to different haptoglobin proteins and three major genotypes, haptoglobin 1/1,2/1, and 2/28. Up to now, reported studies in diabetes have been conducted in individuals with presumed type 2 diabetes9,10.

The aims of study identify the relationship with CVD in diabetes patient in Al-Muthanna provice –iraq.

Materials and Method

Ninety patients include (diabetes patient & CVS patient& DM and CVD patient) participated in the present study as well as twenty apparently healthy people were selected as the control group. All patients and control group ages ranged between (35-85) years old during the period from October - 2013 up to March – 2014.These patients were registered as diabetic and CVD patients in "Diabetic Unit" at "Al-Hussien Hospital" in Al-Muthanna province- Iraq.

PCR assay was performed for detection of haptoglobin genotype directly by amplification using allele specific primers in haptoglobin gene in diabetic and CVD patients. For the diagnosis of specific Hp1 & Hp2 a pair of allele specific primers one of which has its 3′ terminal nucleotide complementary to the haptoglobin genotype

1.Blood Samples

Five ml of venous blood were collected from each person (patients and controls) involved in the present study samples, and the collected blood was divided to (three ml for serum test) and two ml for PCR-test using EDTA polypropylene tube. About 400 ml of the EDTA blood was used for DNA extraction and purification using Genomic DNA mini kit extraction kit (Fresh Blood) Gene aid. USA. The purity and concentration of extracted genomic DNA from blood samples was checked by using Nano drop spectrophotometer (THERMO. USA) by reading tits absorbance at (260 / 280nm).

2.PCR master mix preparation

For each disease, a number of samples are tested according to phenotypes of the disease for both patient and control groups. Two PCR reactions (two tubes)are performed for each sample: one for identifying the presence of the HP1(using the primer A, B) and the other for the presence of HP2 (using the primer C, D). PCR master mix was prepared by using (Amp ONETMTaqPreMix Kit) and this master mix done according to company instructions as explained following table:

ARMS PCR Master mix	Volume
DNA template	1.5µl
Internal Primer A (10pmol)	1µl
Internal Primer B (10pmol)	1µl
Internal Primer C (10pmol)	1µl
Internal Primer D (10pmol)	1µl
PCR water	14.5µl
Total volume	20µl

After that, these reagents were placed in standard AmpONE^{TM} TaqPreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). Then was placed in PCR Thermo cycler (THECHNE.USA).

PCR Thermo cycler Conditions used were shown in table–1; to detect the HP1was adopted.

Table 2-1: PCR program

PCR step	Temp.	Time	Repeat
InitialDenaturation	95C	2min	1
Denaturation	95C	20sec	
Annealing	65C	10sec	35cycle
Extension	72C	2min	
Final extension	72C	5min	1
Hold	4C	Forever	-

To detect the HP2 in PCR a program used was shown in table -2-2:

Table2-2 : PCR program To detect the HP2 was adopted

PCR step	Temp.	Time	Repeat
InitialDenaturation	95C	2min	1
Denaturation	95C	20sec	
Annealing	57C	10sec	35cycle
Extension	72C	2min	
Final extension	72C	5min	1
Hold	4C	Forever	-

Genomic DNA was extracted from peripheral bloodleu-kocytesusingthe QIA amp DNA Blood Kit as suggested by the supplier (Qiagen). Oligonucleotide primers (5GAGGGGAGCTTGCCTTTCCATTG-3)and(GAGATTTTTGAGCCCTGGCTGGT-3)wereused foramplificationofa1757-bpHp1allele-specificsequence and a 3481-bp Hp2 allele-specific sequence Primers C (5-CCTGCCTCGTATTAACTGCACCAT-3) and (5-CCGAGTGCTCCACATAGCCATGT-3)were used to amplify a349-bp Hp 2 allele-specific sequence.PrimersweresynthesizedbyAppliedBiosystems11.In Hp 1 and Hp 2, the annealing sites for primer A arelocatedimmediatelyupstreamofthe1711-bpunitandthe1724-bp unit, respectively Results and Discussion The nucleotide at the 5end of primer A corresponds to position 188639 in AC004682 (Hp 1) and position 2781 in M69197 (Hp 2). PrimerBhasbindingsitesjustdownstreamofthe1711-bp elements of Hp1and Hp2. The nucleotideatthe5 end of primer B corresponds to position 186883 in AC004682 (Hp 1) and position 6261 in M69197 (Hp 2). Depending on the genotype represented by the template DNA,aHp1- specificproductof1757bpand/or aHp2-specific product of 3481bp is generated in PCRs with primers A and B. Primers C and D have one binding site in allele Hp 1and two binding sites in allele Hp 2 .In reactions with primers C and D, a PCR product, 349 bp in length, is generated only in the presence of the Hp 2 template, whereas no product is formed in the presence of the Hp 1 template . This kind of allelic specificity is attributable to the relative positions of the binding sites and the 5 33 orientation of primers C and D. The template for the 349-bp Hp 2-specific amplification product, including the annealing sites for primers C and D, extends from nucleotide position 4352 to 4700 in M69197. There is also one binding site each for primers C and D at the corresponding positions in the haptoglobin- relatedgene, although these quences of primers and an nealingsites are not 100% complementary. Importantly, with the haptoglob in-related gene as a template, amplification reactions with primers C and D proceed in opposite directions, which do not allow a PCR product to be generated.

1.DNA Isolation

The band integrity and DNA concentration were found to be different according to the yielded amount of genomic DNA and its purity depend on the amount of white blood cells in the blood samples. In addition, the use of fresh blood samples was found to be better, therefore, the DNA isolation should be applied as early as possible and DNA concentration in the present study was 10-100 ng.

1-1.PCR Screening

In PCRs with primers A and B (protocol 1), amplification products of 1757 and 3481 bp were amplified from genomic DNA containing alleles Hp 1 and Hp 2, respectively (Fig.1). After electrophoresis of the reaction products in 1% agarose gels, Hp genotype-specific banding patterns were obtained: genotypes Hp 1-1 and Hp 2-2 were characterized by single bands representing the 1757-and 3481-bp products, respectively. In the presence of the 1757- bp product, it was not possible to conclusively determine whether the 3481-bp Hp 2-specific PCR product was also present. In these cases, an alternative protocol (protocol 2), consisting of two separate reactions, was chosen: one reaction, using primers A and B, was aimed at detecting the 1757-bp Hp 1-specific product, and the other reaction, using primers C and D, was aimed at detecting the 349-bp Hp 2-specific product (Fig.2). Using 1% agarose gels,. Importantly, the appearance of the additional products, characteristic of the PCR system using four primers, did not interfere with genotype determination. The 3481-bp Hp 2-specific product was not synthesized in detectable amounts in the presence of the four primers combined. Comparative testing with DNA samplesfrom50 individuals showed that the tow protocols for haptoglob in genotyping yielded identical results. The haptoglobingeno types of 40 consecutive patients were determined with genomic DNA prepared from blood samples of diabetes-heart patient as show in fig (1) withPCRprotocol1.Inthesecases, the1757-bp Hp1-specific product was present as relatively weak b and, probably because of low concentrations of genomic DNA. Inthesamesample,the3481-bpHp2-specificb and was visible, and it was clear in fig(1)while other individual samples did not contain the Hp 2 allele or whether the 3481-bp product was present at concentrations too low to be detected in the gel. But the HP2 349 bp we found it in all 50 sample .this result in protocol 1 and 2 as show in fig(3-2), for all patient type and control.

Figure (3-1): PCR product of HP1 genotype on 1% agarose gel at 100 voltages for one hour. Sample 1: ladder.Sample 2: contains an amplified product HP1(1757 bp) and HP2(3491bp) diabetes – heart patient sample.

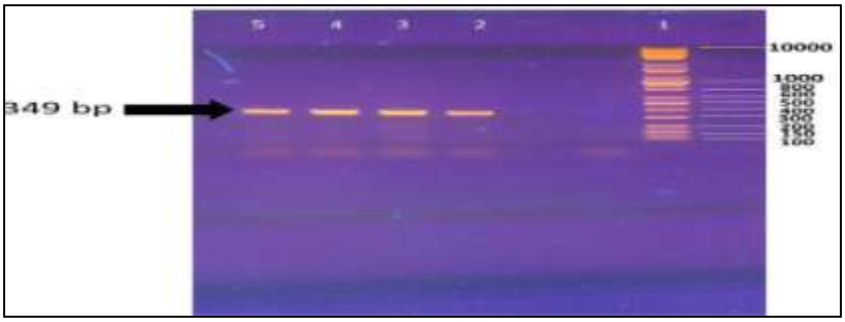

Figure (3-2): PCR product of HP2 genotype on 1% agarose gelat 100 voltages for one hour. Sample 1: ladder.Sample 2: contains an amplified product HP2 in control sample Sample 3: contains an amplified product HP2 in diabetes patient sample Sample 4: contains an amplified product HP2 in heart patient Sample 5: contains an amplified product HP2 in diabetes- heart Patient

In 2013 Jeremy *et al*[13] observed suggestive evidence for association of the *HP* duplication polymorphism with CVD related mortality in the DHS. In addition, so this study found that the *HP 2–2*genotype was associated with T2DM status.

Several prior studies have investigated *HP* polymorphisms and CVD risk in T2DM. In 2002 Levy *et al*[14]. reported CVD events in diabetes five times greater with the HP 2genotype, than with HP 1 in a study that included 206 CVD patients and 206 CVD controls (146 and 93 were affected by T2DM, respectively, as part of the Strong Heart Study). In 2004, a subsequent study by Levy *et al.*[15] included 3273 individuals in the Framingham Heart Study, however only a subset of 433 individuals were affected with T2DM, and of these, only 86 had a history of prevalent CVD. Finally, a 2003 study in individuals with acute myocardial infarction (AMI) reported individuals with T2DM and the *HP2* allele had increased mortality following AMI compared to individuals with T2DM and the *Hp 1* genotype (included only 224 T2DM-affected individuals)[16]. In the present study we detected modest evidence of association with MI, but did not strongly replicate association with history of prior CVD.

IMT and measures of vascular calcification are not highly correlated[17]. The DHS is predominately comprised of T2DM-affected subjects (1013 of 1208 participants). Our primary measures were the subclinical measures of CVD, CAC and IMT which may not be as strongly influenced by *HP* polymorphism. Of the DHS subjects, 435 were T2DM-affected participants with a history of prevalent CVD, based upon self-reported history and prior intervention which was not associated with *HP* genotype. The analysis with CVD mortality, a firm endpoint, suggests a possible contribution to risk. Given the association of the *HP 2* genotype with risk for mortality, it is possible that a survival bias may be present. However, genotype frequencies were consistent with Hardy-Weinberg equilibrium. In addition, the genotype frequencies of the *HP* duplication in this study were similar to those reported previously[18].

In prior reports, two promoter SNPs, rs5470 and rs5471 were associated with altered levels of *HP* expression[19,20] with rs5471 reported to be associated with the Haptoglobin 1–2 modified (HP1-2mod) phenotype. In individuals with the rs5471 "C" allele and the *HP 12* genotype, normal expression levels of the HP 1 protein, has been suggested that the decreased levels of HP 2 lead to greater oxidative stress[21]. Several previous studies have investigated the effect of the HP polymorphism on T2DM risk. A previous study by Stern *et al*. in 1986[22] found that the *HP1* allele was associated diabetes risk in Mexican Americans. They found that a single copy of the *HP1* allele increased T2DM risk by 50% and a second copy increased risk by 100%. A second report from 2006 by Quaye*et al*.[23] found that the HP 2–2 phenotype was a risk factor for T2DM in a population in Ghana. The current study had a larger sample size than either of the two previous studies, albeit in a different ethnicity. In this study it was found that EA individuals with the *HP 2*–2 genotype are more likely to have T2DM with an OR of 1.49. These studies, when combined suggest that *HP* is a risk gene for diabetes or is in LD with a risk gene. Several studies have shown that the different alleles lead to different levels of circulating HP protein [24,25]. Higher circulating HP has been suggested to be associated with metabolic syndrome, high blood pressure, and elevated glucose[26]. This could possibly explain the association of the HP polymorphism with T2DM. Different risk alleles across populations are problematic as it could be difficult to assess risk across different populations.

Importantly, the T2DM association may be difficult to further investigate without subsequent data generation since the duplication is not included in the major, publically available, databases. Furthermore, any subsequent studies will require a targeted phenotyping approach either through analysis of HP in serum or genotyping through fragment size analysis as performed in this study, since the duplication is not captured by the current commercially available genome-wide genotyping platforms and does not appear to be tagged by other common polymorphisms[25].

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الخلاصة

هدفت هذه الدراسة لمعرفة العلاقة بين جين الهابتوكلوبين وامراض القلب الوعائية في مرضى السكري بواسطة اختبار الPCR للمرة الاولى في محافظة المثنى خلال الفترة من اكتوبر (2013) وحتى مارس (2014).

الدراسة شخصت نوعين من جين الهابتوكلوبين بواسطة اختبار الPCR الاول(HP1 1757bp) والثاني (HP2 349bp).