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Biochemistry Section

Assessment of Haptoglobin 2-2 Genotype in Type 2 Diabetes and Cardiovascular Patients in North Indian Population: A Case-control Study

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ABSTRACT

Introduction: Haptoglobin (Hp), an acute phase protein, is a plasma inflammatory marker. It is important in both infectious and non infectious illnesses. When intravascular haemolysis occurs, the dead red blood cells are discharged into the circulation and link to Hp to form the haemoglobin (Hb)-haptoglobin (Hb-Hp) complex that is recognised by the macrophage scavenger receptor CD163. The Hp1 and Hp2 are its two alleles. The Hp2 is formed by a partial duplication of the Hp1 gene. The Hp1 has five exons, but Hp2 has seven exons owing to the duplication of Hp1 third and fourth exon. There are three genotypes of Hp: Hp1-1, Hp2-1, and Hp2-2. The encoded products of genotypes are attached to haemoglobin with varying degrees of affinity: Hp1-1 has the most affinity, Hp2-1 has medium affinity, and Hp2-2 has the lowest affinity. Hp allelic and genotypic variation differs amongst populations and different geographic regions of the world.

Aim: To study the haptoglobin 2-2 genotype in type 2 diabetes and cardiovascular patients in north Indian population.

Materials and Methods: It was a case-control study and a total of 200 participants were recruited randomly including 50 participants of type 2 Diabetes Mellitus (DM), 50 participants of Cardiovascular Disorders (CVD), 50 participants with Cardiovascular Disorders With type 2 Diabetes Mellitus (CVDWDM) and 50 normal healthy controls from Punjab Institute of Medical Sciences (PIMS) Hospital and clinics and Aashirwad laboratory of Jalandhar, Punjab, India in February 2021 to January 2022. Anthropometric variables were determined by using standard methods and biochemical parameters were measured by commercial available kits. Allele specific Polymerase Chain Reaction (PCR) or Amplification Refractory Mutation System (ARMS) PCR was used to detect Hp genotypes. Data were represented as mean±Standard Deviation and the statistical difference i.e. $p<0.05$ between all parameters was determined by using the Mann-Whitney U test.

Results: Anthropometric parameters i.e. Body Mass Index (BMI) was found to be statistically significant in CVDWDM as compared to CVD, controls and T2DM i.e. p=0.023, p=0.01 and p=0.014. Waist Circumference (WC) was found to be statistically significant in CVDWDM as compared to CVD, controls and T2DM i.e. p=0.02, p=0.013 and p=0.012 and Hip Circumference (HC) was found to be statistically significant in CVDWDM as compared to CVD, controls and T2DM i.e. p=0.02, p=0.017 and p=0.014. However, Apolipoprotein A1 mean value was found to be higher and statistically significant i.e. p=0.03 in Type 2 Diabetes Mellitus (T2DM) as compared to healthy participants. Triglycerides mean value was increased in T2DM as compared to CVD, CVD as compared to controls and CVDWDM as compared to healthy controls (p-value=0.012, 0.04, 0.015, respectively). The findings had shown that a 349bp band was detected in patients with T2DM, CVD and CVDWDM, implying that the Hp2-2 genotypes were observed but not in healthy controls.

Conclusion: The study finds that people with DM, CVD, or CVDWDM all have a 349bp band. It means that the genotypes of the Hp2-2 allele were found in patients of diabetes, CVD and diabetics with CVD but not in the healthy individuals.

Keywords: Allele, Apolipoprotein, Glycosylated haemoglobin, Polymerase chain reaction

INTRODUCTION

Diabetes is a non communicable disease that is ravaging both industrialised and developing nations. In 2011, 366 million individuals were impacted globally, and this number is expected to climb to almost 552 million by 2030 [1]. Diabetes is a chronic, and life threatening metabolic illness brought on by a high blood glucose level in the body. Type 2 Diabetes Mellitus progresses slowly and is difficult to detect in its early stages [2]. One of the most serious outcomes of T2DM is CVD. Similarly, throughout the last two to three decades, a CVD epidemic has emerged throughout the world. In 1990, it was estimated that 5.3 million fatalities were caused by CVD in industrialised nations, while the comparable numbers for underdeveloped nations were between 8 and 9 million (i.e. a relative excess of 70%) [3]. Moreover half of those with T2DM experience a coronary artery disease, a stroke, or a heart attack [4]. Hp is a glycoprotein produced in the liver in response to cytokines including IL-1, IL-6, and TNF (Tumour Necrosis Factor). Hp is a protein that looks like an immunoglobulin in which disulfide bridges connect two alpha (light) and two beta (heavy) chains (S-S). The Hp gene is found on chromosome 16q22.1, which is the long arm of the chromosome [5]. The Hp1 and Hp2 are the two autosomal co dominant alleles. Hp1 has five exons, and Hp2 allele was created by the duplication or multimerisation of exons 3 and 5 of Hp1, in total there are seven exons in this gene [6-8].

In diverse ethnicities and ethnic groups across the world, there are allelic and genotypic differences in Hp. Studies have reported that in India, (Maharashtra, Rajasthan, Delhi and Uttar Pradesh) the allelic frequency of Hp was measured by using starch gel electrophoresis, polyacrylamide gel electrophoresis, and the Enzyme Linked Immunosorbent Assay (ELISA) technique from healthy people in order to conduct a caste-based study on control subjects [9-15]. In India, there is no work on the molecular level related to Hp gene. For the above study, this is the first extensive genotypic analysis in Northern India. Some studies have shown that the Hp2-2

genotype raises the possibility of coronary diseases [16,17]. The study hypothesised that, CVD is substantially more likely to occur in diabetics who are homozygous for the haptoglobin 2 genotype (Hp2-2). The impact of Hp2-2 protein in both cardiovascular and diabetes diseases was being investigated for the first time in the present study.

MATERIALS AND METHODS

The current case-control study included 200 people randomly (convenience sampling) and was carried out from February 2021-January 2022. Out of 200, 50 were having T2DM, 50 were CVD, 50 were CVDWDM, and 50 were healthy controls. The research work for the current study was assessed and approved by the Institutional Ethics Committee of Lovely Professional University, Phagwara, (LPU/IEC/2018/01/01). After providing their informed consent, all individuals were included from the clinic.

Inclusion criteria: The patients diagnosed with diabetes mellitus on the basis of American Diabetes Association criteria (ADA, 2015) on the basis of glycated haemoglobin (A1C) ≥6.5% and aged 26-75 years, were enrolled (males and females) from Northern India. Healthy individuals without history of any chronic disease were included in the present study [18,19].

Exclusion criteria: Patients with a history of diabetic retinopathy, gestational diabetes was excluded from the study. Pregnant ladies, nursing mothers and with age <26, and >75 years and children were excluded from the study.

Study Procedure

Anthropometric measures were obtained when the individuals were dressed in light clothes and were not wearing shoes. A calibrated stadiometer and a portable weighing machine were used to determine height and weight. A non stretchable plastic tape was used to measure the circumference of the hips. The minimal horizontal girth between the costal margins and the iliac crests at the conclusion of normal expiration was used to calculate waist circumference. Waist-Hip Ratio (WHR) was determined by waist circumference divided by hip circumference. The BMI was calculated by the weight in kilogrammes (kg) by the squared height in metres (m^2) [20]. Each participant's fasting venous blood (5 mL) was collected into Ethylenediamine Tetra-acetic Acid (EDTA)-coated vials, and then centrifuged at 4500 rpm for 5 minutes. The resulting supernatant or serum were collected into two 1.5 mL micro-centrifuge tubes or vials and stored at -20°C for further biological investigation [21].

Biochemical Analysis

A typical glucometer gadget was used to measure Fasting Blood Glucose (FBG) levels [22]. Total cholesterol, triglycerides, Apolipoprotein A1 (APOA1) and glycosylated haemoglobin (HbA1c) were measured by using conventional Quantia kits i.e. quantitative turbidimetric immunoassay technique manufactured by Coral Clinical Systems a division of Tulip Diagnostics (P) Ltd. High Density Lipoprotein (HDL) was calculated by Friedewald formula: HDL=Cholesterol/5, Very Low Density Lipoprotein (VLDL) was calculated by Friedewald formula: VLDL=Triglyceride/5 and Low Density Lipoprotein (LDL) was calculated by Friedewald formula: LDL=TC-HDL-TG/5 [22,23].

Haptoglobin Genotyping

The inorganic technique was used to isolate genomic DNA from whole human blood samples [24]. The genotyping of haptoglobin was performed using the PCR method, as published by Koch W et al., with minor modifications [25]. Each amplification was carried out by using 50 ng of Deoxy Ribonucleic Acid (DNA), i.e. 1 µL in a volume of 20 µL involve 100 µM of primers C and D, 8 µL of PCR nuclease water and 10 µL of PCR master mix. After an initial denaturation at 94°C for 3 minutes, the PCR was run for a total of 30 cycles, with each cycle consisting of denaturation at 94°C for 30 seconds, annealing at 69°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. Total 1.5% agarose gel electrophoresis was used to separate the PCR products. The amplification was found in a 349bp product that was specific to the Hp2-2 allele compared with 100 kb DNA ladder [26].

STATISTICAL ANALYSIS

The data was statistically evaluated with the Statistical Package for Social Science (SPSS) computer application (version 22.0). The data were shown as a mean and Standard Deviation (SD). For abnormally distributed data, the Mann-Whitney U test was used to make comparisons between groups. Statistical significance was determined to exist, when the p-value was less than 0.05.

RESULTS

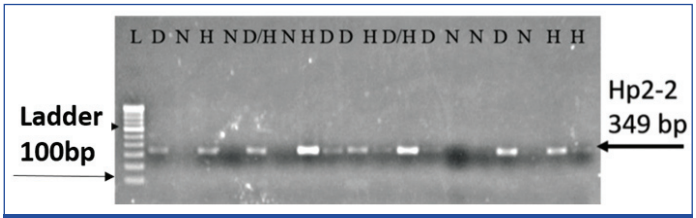
In the present case-control study, 200 volunteers participated. The mean±SD and p-value were measured by Mann-Whitney U test because data were abnormally distributed. The basic demographic data of all participants as shown in [Table/Fig-1], the mean age of T2DM was (52.9±10.733), CVD was (50.92±11.761) and CVDWDM was (55.72±11.471) and in healthy controls was (45.46±13.45). Smoking, Systolic Blood Pressure (SBP), alcoholism, tobacco takers and non takers, dietary habits and duration of T2DM, CVD and CVDWDM, have been represented in percentage. [Table/Fig-2] shows the anthropometric and biochemical parameters. Among anthropometric parameters, the mean value of BMI in CVDWDM was found to be higher and statistically significant i.e. p-value <0.05 as compared to CVD and T2DM patients. The mean value of WC and HC in T2DM was found to be higher and statistically non significant i.e. p-value >0.05 as compared to CVD. The mean value of HbA1c and FBG in T2DM was found to be lower and statistically non significant i.e. p>0.05 as compared to CVDWDM. The lipid profile parameters i.e. TC and HDL were statistically non significant i.e. p>0.05 in all patients as compared to healthy controls. The TG and VLDL were statistically non significant i.e. p>0.05 in CVDWDM as compared to CVD and healthy controls. The mean value of APOA1 was found to be higher and statistically significant i.e. p<0.05 in T2DM patients as compared to healthy controls. Out of 200 participants, the high aspect of the extracted DNA utilised in PCR reaction obtained one hundred fifty 349bp Hp2-2 allele products, of which 50 were diabetics, 50 were having CVD, and 50 were cardiovascular with diabetic patients as shown in [Table/Fig-3].

Variables		T2DM	CVD	CVDWDM	Control
Age (years)		52.9± 10.733	50.92± 11.761	55.72± 11.471	45.46± 13.45
Gender	Male	20 (40%)	30 (60%)	39 (78%)	39 (78%)
	Female	30 (60%)	20 (40%)	11 (22%)	11 (22%)
Smoking	Smokers	32 (64%)	15 (30%)	7 (14%)	-
	Non smokers	18 (36%)	35 (70%)	43 (86%)	-
Tobacco use	Takers	30 (66%)	9 (18%)	5 (10%)	-
	Non takers	5 (10%)	41 (82%)	45 (90%)	-
Dietary habits	Vegetarian	40 (80%)	22 (44%)	21 (42%)	18 (36%)
	Non vegetarian	10 (20%)	28 (56%)	29 (58%)	32 (64%)
Alcohol consumption	Takers	36 (72%)	27 (54%)	27 (54%)	-
	Non takers	14 (28%)	23 (46%)	23 (46%)	-
Physical activity (Moderate)		26 (52%)	27 (54%)	20 (40%)	24 (48%)
SBP (mmHg)	≤140	3 (10%)	19 (60%)	21 (76%)	1 (2%)
	<140	47 (90%)	31 (40%)	29 (24%)	49 (98%)

[Table/Fig-1]: Basic demographic data of the cases and controls.

Parameters	T2DM (N=50)	CVD (N=50)	CVDWDM (N=50)	Normal (N=50)	p-value
BMI (kg/m^2)	26.14±5.88	26.68± 5.36	29.66± 7.04	26.92± 4.88	p1=0.424, p2=0.023, p3=0.964, p4=0.01, p5=0.014, p6=0.414
WC (cm)	37.74± 4.44	37.54± 4.22	40.48± 4.75	35.2± 3.91	p1=0.689, p2=0.02, p3=0.01, p4=0.013, p5=0.013, p6=0.012
HC (cm)	40.48± 4.71	39.7± 4.14	42.6± 4.84	37.2± 3.91	p1=0.409, p2=0.02, p3=0.017, p4=0.017, p5=0.014, p6=0.013
WHR (cm)	0.93± 0.055	0.94± 0.015	0.949± 0.015	0.945± 0.006	p1=0.011, p2=0.012, p3=0.07, p4=0.011, p5=0.014, p6=0.045
FBG (mg/dL)	172.34± 79.34	116.5± 72.98	184.91± 75.41	90.24± 10.46	p1=0.012, p2=0.014, p3=0.011, p4=0.012, p5=0.329, p6=0.017
HbA1c (mg/dL)	8.26± 3.30	6.30± 3.19	7.65±1.70	6.3084 ±3.19	p1=0.012, p2=0.012, p3=0.023, p4=0.013, p5=0.828, p6=0.015
TC (mg/dL)	199.54± 56.32	187.85± 56.29	190.89± 53.53	187.85± 56.29	p1=0.263, p2=0.86, p3=0.508, p4=0.329, p5=0.331, p6=0.064
HDL (mg/dL)	39.90± 11.26	37.57± 11.25	38.17± 10.70	38.17± 10.70	p1=0.26, p2=0.860, p3=0.52, p4=0.321, p5=0.34, p6=0.063
LDL (mg/dL)	78.41± 68.24	115.43± 44.29	115.19± 43.16	89.36± 49.094	p1=0.012, p2=0.76, p3=0.017, p4=0.01, p5=0.017, p6=0.823
TG (mg/dL)	406.09± 280.9	174.24 ±68.37	187.61± 94.03	267.86± 0.40	p1=0.012, p2=0.78, p3=0.04, p4=0.085, p5=0.012, p6=0.015
VLDL (mg/dL)	81.21± 56.18	34.84 ±13.67	37.52 ±18.80	53.57 ±39.42	p1=0.03, p2=0.733, p3=0.03, p4=0.074, p5=0.03, p6=0.04
APOA1 (mg/dL)	118.92 ±74.31	122.06 ±85.51	95.84± 54.005	87.32 ±42.37	p1=0.76, p2=0.260, p3=0.131, p4=0.749, p5=0.069, p6=0.03

[Table/Fig-2]: Anthropometric and biochemical parameters.
p1=T2DM versus CVD; p2=CVDWDM versus CVD; p3=CVD versus N; p4=CVDWDM versus N; p5=T2DM versus CVDWDM; p6=T2DM versus N. p-value <0.05 is statistically significant; TC: Total cholesterol; FBG: Fasting blood glucose; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol; VLDL: Very low density lipoprotein



[Table/Fig-3]: The amplification products of Hp2-2 genotype: H, D/H and D representing Hp2-2 (349bp) band and L representing 100bp to 1000bp DNA ladder. Where, N stands for normal; H stands for cardiovascular patients; D/H stands for cardiovascular with type 2 diabetes; D stands for type 2 diabetes mellitus patients.

DISCUSSION

Despite major breakthroughs in diabetes prevention and medical care, CVD continues to disproportionately afflict diabetics across the world. As a result, the development of vascular problems in diabetes is clearly influenced by genetic susceptibility, and a functional variation in the Hp gene has been identified as a potential indicator of the chance of developing these issues. Early discovery of the condition and prompt treatment can lessen the disease's morbidity and death [27,28]. In the present study findings the anthropometric variables i.e. BMI was found to be statistically significant higher in CVDWDM as compared to CVD and T2DM patients. Similar findings were found in the research conducted by Riaz S and Alam SS; and Sheriff DS et al., Insulin resistance, the main cause of type 2 diabetes, may be contributing to the rise in BMI. Insulin sensitivity of cell membranes is significantly decreased. As a result, the crucial mechanism by which insulin helps glucose flow through the cell wall to be turned into energy is significantly hampered. As a result, extra glucose continues to circulate in the circulation, resulting in high blood sugar levels that are transferred to the liver. Once there, the sugar is transformed into fat and circulated throughout the body via the blood stream. Obesity and weight increase are outcomes of this mechanism [23,29].

The results of WC and HC were not found to be similar with Riaz S and Alam SS, the BMI and WHR measurements are dependent on dietary patterns and dwindling levels of physical activity. It has been hypothesised that, these environmental influences, particularly in those people with metabolic genotype, reveal a hereditary vulnerability to obesity [23]. The results of TC, HDL, TG and VLDL were not found to be similar with Sheriff DS et al., [29]. The LDL was statistically non significant i.e. p>0.05 in T2DM as compared to healthy controls and similar finding was found in the research conducted by Sheriff DS et al. According to reports, VLDL and LDL absorption in the liver is reduced in T2DM patients, which causes their amounts of these lipoproteins in the plasma to rise, especially in the postprandial period. This condition is most frequently seen in type T2DM patients who have severe insulin insufficiency or inadequate glycemic control. Additionally, it has been stated that the lower availability of LDL receptors contributed to the restricted LDL clearance [29,30]. Previous research has connected DM and diabetic microvascular problems, such as nephropathy and retinopathy, to the Hp polymorphism, piquing researchers' interest in elucidating the function of Hp phenotypes in DM and related cardiovascular consequences [7].

The goal of the present study was to gather information on Hp2-2 and type 2 diabetes, CVD and CVDWDM in North Indian population in order to see if the Hp2-2 are linked to the development of CVD in diabetes. This investigation revealed the existence of Hp2-2 genotype in T2DM, CVD and CVDWDM participants, but not in the case of healthy participants. The role of Hp in oxidation and inflammation has sparked speculation about its possible link to vascular disease, particularly in the presence of elevated oxidative stress [16]. It has been reported that, diabetic people with the Hp2-2 phenotype have a five-fold higher chance of having CVD than people with the Hp1-1 phenotype. It was discovered that the

Hp2-1 phenotype was connected to an intermediate risk of CVD. Individuals without diabetes mellitus, however, did not show any connection [27]. The Hp2-2 genotype was linked to a very significant increase in the risk of severe adverse cardiac events in a study, on 935 DM patients [31]. Firstly, the Hp2-2 protein molecule stays in the transmission for a longer period of time and is shown to have undergone greater oxidative damage owing to the bigger molecule of Hp1-1 [32]. Secondly, in the Hp1-1 protein molecule haem variation from methemoglobin (metHb) to LDL molecule was removed but partially occurs in Hp2-2. Due to that limited haem transfer from the Hb-Hp2-2 complex to LDL causes oxidation of LDL lipids, as well as, protein. The lower capability of Hp2-2 protein to preserve haem molecule in the haem pocket of HbA1c [16]. Hence, Hb-Hp2-2 molecules in diabetes are removed more slowly than the Hb-Hp1-1 complex. When the participants have both Hp2-2 genotype and HbA1c ≥6.5%, they may be at greater risk of CVD from this increase in plasma redox-active Hb-Hp complex. This complex is further linked to HDL molecule, thus, results in damage to the activity of HDL in stimulating Reverse Cholesterol Transport (RCT), decrease antioxidant activity, and increase lipoprotein oxidation from heme transport [33]. The APOA1 was found statistically significant i.e. $p<0.05$ in T2DM patients as compared to healthy controls. ApoA-I undergoes post-translational modifications, including fatty acid acylation and oxidation, which have been linked to the environment and metabolism. Non enzymatic protein glycation and Advanced Glycation End Product production have received a lot of interest in diabetic patients. Both are linked to hyperglycemia in type 1 and type 2 diabetes, where AGE adducts bind to ApoA-I and prevent it from activating Lecithin Cholesterol Acyltransferase (LCAT), the enzyme that, converts nascent HDLs to mature HDLs. The development of Coronary Artery Disease (CAD) appears to be accelerated by AGEs. Modification experiments on ApoA-I have also revealed that the most basic versions are the protein's less mature isoforms. In most forms of amyloidosis, atherosclerosis, and neurodegenerative disorders, proteolysis is assumed to play a key role. In fact, HDL-associated ApoA-I is really destroyed by macrophage metalloproteinase in coronary patients at both the N and C termini. As a result, the transition from lipid-free ApoA-I to spherical HDL particles, may be hampered by the lack of the first 38 amino acids present in ApoA-I (1-38). Possibly decreasing reverse cholesterol transit and making it easier for it to attach to LDL particles. Large HDL complexes need the N-terminal amino acid residues, and their removal results in less stable HDL particles [34,35]. The Hp2-2 genotype is assessed in patients with diabetes and related consequences. Therefore, the Hp2-2 genotype may be a valuable indicator of a person's likelihood of developing DM and its consequences [36]. The low cost and ease of use of the genotyping technique, are another advantage of the present study. Another application is to detect Single Nucleotide Polymorphisms (SNPs), small insertions/deletions, and copy number variations are another benefits of genotyping approaches over whole genome sequencing and genome wide association studies (which is the case for haptoglobin).

Limitation(s)

In the present study, the variations between Hp1-1 and Hp1-2 individuals were not examined. The sample size was less, therefore, there was lack of correlation in demographic data of participants. This was a further drawback of the present cohort, and future research should determine the precise sample size, required for detecting possible variations among all three haptoglobin genotypes.

CONCLUSION(S)

The study concluded that, a 349bp band was seen in diabetes patients, cardiovascular patients, and in diabetics with CVD. It indicated that, the Hp2-2 allele genotypes were discovered in these patients but not in healthy people in the population. The Hp2-2 genotype is a CVD risk factor in type 2 diabetic patients in the North Indian population, as per the present study's findings. The data showed that, Hp genotype may be used to stratify patients for optimal CVD therapy in people, with type 2 diabetes.

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