Haptoglobin Polymorphism and Association with Complications in Ghanaian Type 2 Diabetic Patients

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Abstract There is scanty information on the role of genetic factors, especially those relating to haptoglobin (Hp) phenotypes in the expression of complications among diabetes mellitus patients in Ghana. In this study, we investigated whether there is any association between Hp phenotypes and diabetic complications and to determine if association of the Hp phenotypes with diabetic complications in Ghanaian diabetics differ from those in Caucasians. A total of 398 participants were randomly recruited into the study. These comprised diabetic patients numbering 290 attending a diabetes Clinic in Ghana and 108 non-diabetic controls from the same community. Analyses of the results indicate that most of the diabetics with complications were of the Hp 2–2 (35%) and Hp 2–1 (23.9%) phenotypes. Fewer diabetics were found to be of the Hp 2–1 M

phenotype. The controls were mostly of Hp 1–1 and Hp 2–1 M phenotypes. The odds ratio of having complications in a diabetic with an Hp 2–2 phenotype was 18.27 times greater than that for Hp 0–0. Hp 2–2 phenotype with its poor antioxidant activity may therefore be a useful predictor for the propensity of an individual to develop diabetes complications.

Keywords Diabetes mellitus · Antioxidants · Haptoglobin phenotype

Background

Haptoglobin (Hp) is an acute phase protein which forms 1% of the total plasma mass. Hp is present in most body fluids such as serum, urine, saliva, amniotic fluid and ascites. It is synthesized mainly by the hepatocytes in the liver [1, 2] and to some extent, non-hepatic cells including adipocytes, spleen of mammals and lung cells in response to interleukin-6 (IL-6) type cytokines and tumour necrosis factor- α (TNF- α) cytokines [3]. In humans, there is a genetic polymorphism in the expression of Hp. A common polymorphism of the Hp gene, which is located on the long arm of chromosome 16 (16q22.3) [4] is characterized by two common co-dominant alleles; Hp α^1 and Hp α^2 [5]. These give rise to structurally and functionally distinct Hp phenotypes namely Hp 1–1, Hp 2–1 and Hp 2–2 [6, 7].

A number of additional Hp phenotypes have been reported in blacks, namely Hp 0–0 and Hp 2–1 M [8, 9]. The genetic basis for Hp 0–0 is not clearly defined even though it has been stated from a study in Japan, China and Korea that an α -chain deletion in the promoter region of an individual was shown to cause ahaptoglobinaemia [10, 11]. According to Teye et al. [12], the genetic basis of Hp 0–0

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in Ghana is different from that seen in Asia. They found that a –61A–C base substitution in the promoter region of the Hp gene was associated with ahaptoglobinaemia and was strongly associated with the Hp α^2 allele. The Hp 2–1 M phenotype is prevalent in West African populations and Black Americans with West African ancestry [9] and it is associated with a decreased Hp 2 gene expression [13]. Hp 2–1 M results when the amount of Hp 2 polypeptide synthesized in the Hp 2/Hp 1 heterozygotes is reduced. The variability seen in the Hp 2–1 modified phenotype, which ranges from close to Hp 2–1 to close to Hp 1–1, can be explained in part by the existence of several Hp 2 alleles differing in their promoters and possibly in part by differences in the promoters of the accompanying Hp 1 allele [13].

There exist functional differences among the various Hp phenotypes. These functional differences invariably associate Hp polymorphism with the prevalence and clinical evolution of many autoimmune and inflammatory diseases, including severity of myocardial infarction [14, 15]. Hp functions as an antioxidant by virtue of its ability to bind and capture free haemoglobin released into blood plasma [16, 17] and thereby prevents the oxidative tissue damage that may be mediated or catalysed by free haemoglobin through the generation of highly reactive oxygen species by the Fenton reaction [18]. Formation of the Hp-Hb complex in vivo has been found to play a role in preventing the Hb-driven generation of hydroxyl radical and lipid peroxidation in areas of inflammation where reactive oxygen species (ROS) promotes endothelial activation and inflammation, leading to endothelial dysfunction [19].

In both type 1 and type 2 diabetes mellitus (DM), complications in target organs arise from oxidative stress resulting primarily from chronic hyperglycaemia [20, 21]. The generation of this oxidative stress [22] may be moderated by exogenous and host genetic factors that scavenge free radicals. Endogenous antioxidants such as glutathione and certain haptoglobin (Hp) phenotypes are important in the prevention and protection of blood vessel walls of internal organs against oxidative stress which is normally increased in the diabetic state by the presence of glycated haemoglobin [23].

A number of diabetic vascular complications have been associated with different phenotypes of haptoglobin [24]. An earlier study by Adinortey et al. [25] reported of association between Hp phenotypes and diabetic retinopathy among Ghanaians. Elsewhere, Hp 2–2 is known to be associated with diabetic vascular complications such as nephropathy, neuropathy and hypertension [26]. It is unknown in Ghana whether there is any association between diabetic complications and Hp phenotypes. The main objective of this study was to investigate if there is any association between Hp phenotypes and diabetic

complications and also determine if a specific Hp phenotype could potentially identify diabetic patients at increased risk for these complications.

Methodology

Informed Consent and Ethical Clearance

The Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana examined and approved the research proposal for this study and each participant provided written informed consent and completed a questionnaire.

Recruitment of Study Participants

The study was a cross sectional one. Sample size was calculated using 5% level of significance, a power of 80%, and an Hp 2–2 prevalence of 3% in normal Ghanaians and 24% in diabetics [27]. A total of 398 participants were recruited into the study comprising type-2 diabetic patients attending the Diabetes Clinic of the Tema General Hospital and healthy non-diabetic controls from the Accra-Tema metropolis of Ghana.

Clinical Assessment

Each diabetic patient or non-diabetic control, after granting his or her informed consent, was given a full physical examination by a physician at the Tema General Hospital. The clinical history of each patient including (a) demographic data and (b) disease condition and treatment history was also recorded. Participants were weighed in light clothing without shoes and their heights were measured with a stadiometer. The mean of two blood pressure (BP) readings, measured on the right arm after participants had been seated for 5 min, was recorded. Body Mass Index (BMI) was calculated as kilogram per metre square.

Diagnosis and Classification of Cases

In this study, type-2 DM was diagnosed using the World Health Organization Criteria [28]. Diabetic complications were diagnosed based on full physical, clinical and laboratory examination by a specialist. Overt nephropathy (proteinuria) was defined and diagnosed based on the criteria that a patient repeatedly had either a urinary albumin excretion rate of >200 $\mu\text{g}/\text{min}$ or a positive urinalysis for protein using a reagent strip [29, 30]. The definitions adopted in assessing Hypertension were: systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg or participants currently receiving treatment for hypertension [31]. Diagnosis of retinopathic

patients was done using ophthalmoscope in a darkened room with the pupil dilated [32]. Normal hepatic function was indicated by levels of AST and ALT. Those excluded from the study were non-diabetics with proteinuria (overt nephropathy), neuropathy, retinopathy, hypertension, acutely ill patients, and pregnant women to avoid a false proteinuria. Those included were already diagnosed and newly diagnosed type-2 diabetic patients with fasting blood glucose (FBG) ≥ 6.4 mmol/l.

Urine and Blood Sampling

The first morning urine specimens were collected from all participants into clean well labelled bottles. Protein in urine was determined immediately using urine reagent strips (URO PAPER-Japan). The results were reported according to the colour chart provided by the manufacturer. Fasting venous blood (5 ml) samples were collected from each participant into EDTA free tubes, allowed to clot for 30 min, and centrifuged at 4500 rpm for 5 min using Hitachi 20PR-52D. The supernatant fractions (sera) obtained were aliquoted into two 1.5 ml eppendorf tubes and stored at -20°C for later biochemical analysis and haptoglobin electrophoresis.

Biochemical Measurements

Fasting Blood Glucose was determined using enzymatic oxidation in the presence of glucose oxidase (GOD) [33]. Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) levels were also determined using method described by Reitman, and Frankel [34].

Haptoglobin Phenotyping

Haptoglobin phenotypes of haemoglobin-supplemented plasma, were determined according to the method described by Giblete [35]. For electrophoresis, 6 μl of serum samples was incubated with 1 μl of 10% haemolysate of washed human red blood cells to enable the Hb content of the haemolysate to form Hp-Hb complex, after which 6 μl of loading buffer was added to the reaction mixture. Then 8 μl of each reaction mixture was loaded for electrophoresis according to the method described by Davis [36] after which proteins were fixed using 10% trichloroacetic acid solution and stained with benzidine solution. The bands were then observed for Hp phenotyping.

Statistical Analysis

Results collected from diabetics with or without complications, and non-diabetics (controls) were presented as percentages and the mean $\pm$ SEM (standard error of the

mean) on a table. The distribution of Hp phenotypes was represented using bar graphs. The association between Hp phenotypes and diabetic complications were tested using Pearson's chi-square tests. Logistic regression analysis was conducted at the 5% level of significance (SPSS 16.0) to determine diabetics' Hp phenotype relative risk of developing complications.

Results

The volunteers in the study comprised two hundred and ninety (290) type-2 DM individuals and one hundred and eight (108) non-diabetic individuals. The major complications recorded in the study were nephropathy, retinopathy, neuropathy and hypertension. From the results, 71% of the two hundred and ninety (290) diabetics recruited for the study had at least one of the listed complications. Out of the total of 290 diabetics, 28% had neuropathy, 17% had both nephropathy and hypertension and diabetics with retinopathy constituted 9% and the remaining 29% showing no form of complication.

Table 1 shows some of the results obtained. The Table shows that there were more female diabetics (65.2%) than male diabetics (34.8%). There were more apparently healthy female volunteers than males. The male diabetics were significantly older than the females ($P < 0.05$); however, there was no statistical difference between the ages of the male and female non diabetic volunteers ($P > 0.05$).

The Table also shows that the BMI of diabetic patients was significantly higher than that of the non-diabetic subjects ($P < 0.05$). For both groups, the females had higher BMI values ($P < 0.05$). The mean systolic and diastolic blood pressures were significantly higher for the diabetic groups compared to the non-diabetic group ($P < 0.05$) but no sex differences were observed ($P > 0.05$) (Table 1). The mean FBG level for diabetics was significantly higher than that for the non-diabetic groups ($P < 0.05$). Similarly, no sex differences were observed in the mean FBG levels among the groups. No statistically significant differences were observed in AST and ALT levels among the participants ($P > 0.05$).

Figure 1 shows that 35% of the diabetics were of the Hp 2–2 phenotype compared to 9% of the non diabetics. Conversely, for Hp 1–1 phenotype, there were twice as many non-diabetics as there were diabetics in the group ($P < 0.05$). Diabetics outnumbered the non-diabetics in the Hp 2–1 group whereas the opposite was true for the Hp 2–1 M group. Only a few of the participants, 8.3 and 6.2% of controls and diabetics, respectively, were of the Hp 0–0 phenotype. Statistical analyses did not reveal any significant sex difference in the Hp distribution.

Table 1 Clinical and biochemical indices of non-diabetics and diabetics

Parameter	Participant				Ref. range
	Non-diabetics		Diabetics		
	Male	Female	Male	Female	
No. of persons	46	62	101	189 ^c	
Age (Years)	52.70 ± 1.49	51.27 ± 1.48	56.83 ± 0.97	53.36 ± 0.74 ^c	
BMI (Kg ^m ⁻²)	23.66 ± 0.28	25.07 ± 0.42 ^b	26.86 ± 0.51	28.58 ± 0.40 ^{a c}	18.8–24.9
Systolic (mmHg)	118.48 ± 0.82	118.00 ± 0.77	130.19 ± 1.47	128.94 ± 1.00 ^a	≤120
Diastolic (mmHg)	77.39 ± 0.66	76.77 ± 0.60	85.94 ± 1.05	84.07 ± 0.62 ^a	≤80
FBG (mM)	4.99 ± 0.094	5.45 ± 0.22	9.65 ± 0.37	9.24 ± 0.28 ^a	4.2–6.4
AST (U/l)	8.97 ± 0.42	9.28 ± 0.32	9.39 ± 0.43	9.09 ± 0.22	Up 12
ALT (U/l)	7.56 ± 0.30	8.04 ± 0.31	8.61 ± 0.42	8.09 ± 0.20	Up 12

Significant difference ($P < 0.05$) between **a** diabetics and non-diabetics; **b** non-diabetic males and females; **c** male and female diabetics

Fig. 1 Percentage distribution of Hp phenotypes among diabetics and non-diabetic participants

Among the diabetic participants, 25 individuals were found to have retinopathy. None of the diabetics with Hp 2–1 M phenotype had retinopathy. However, 6 out of the 25 (24%) diabetics with Hp 2–1 and 14 of the 25 (56%) diabetics with Hp 2–2 phenotypes had retinopathy. Retinopathy was detected in 16 and 4%, respectively of diabetics with Hp 1–1 and Hp 0–0 retinopathy. Thus, among the Hp phenotypes with retinopathy, there was significantly higher prevalence of Hp 2–2 compared to those with Hp 2–1 ($P < 0.05$). These are displayed in Fig. 2.

The number of diabetics diagnosed to have neuropathy was 82. Figure 2 shows that only 3/82 (3.7%) and 13/82 (15%) of diabetics with Hp 2–1 M and Hp 1–1, respectively had diabetic neuropathy. Out of 82 diabetics, 33 (40%) with Hp 2–2 and 30 (37%) with Hp 2–1 had neuropathy. The difference in the incidence of neuropathy among diabetic patients with Hp 2–2, Hp 1–1 and Hp 2–1 M was statistically significant ($P < 0.05$).

Nephropathy was found to be present in 48 diabetic participants out of which 26(54%) were of the Hp 2–2

Fig. 2 Distribution of Hp phenotypes among diabetics with and without complications

phenotype. Nephropathy was found in 10 (21%) of Hp 2–1, while 3(6%) of them were of the Hp 2–1 M and 8(17%) had the Hp 1–1 phenotype (Fig. 2). There was a significantly higher prevalence of nephropathy in diabetics with the Hp 2–2 and Hp 2–1 compared with Hp 1–1 and Hp 2–1 M ($P < 0.05$).

Fifty [50] of the diabetic patients were also found to have hypertension out of which 23(46%) were of the Hp 2–2 phenotype, 8(16%) had Hp 2–1, 12(24%) were of the Hp 1–1 and 4(8%) were of the Hp 2–1 M phenotype ($P < 0.05$). Thus, among those with diabetes, Hp 2–2 individuals registered the highest prevalence of hypertension (Fig. 2).

Table 2 shows that Hp 2–2, Hp 1–1 had 18.27, and 4.54 times greater risk of developing complications, respectively ($P < 0.01$).

Table 2 Hp Phenotype as a relative risk factor for complications development among diabetics

Risk factor	Odds ratio	CI 95%	<i>P</i> value
Hp 2–2	18.27	6.6–50.58	<0.01
Hp 2–1	4.54	1.73–11.87	<0.01
Hp 1–1	1.39	0.54–3.58	>0.05
Hp 2–1 M	0.58	0.20–1.75	>0.05

Hp 0–0 was used as reference in calculating odds ratio

Discussion

Diabetes mellitus is acknowledged to be on the ascendancy in Ghana [37]. DM is no longer restricted to the ageing population since young adults and children are also being diagnosed with the disease [38]. Early detection of the disease and timely interventions can reduce the morbidity and mortality associated with it. An earlier study by Quaye et al. [27] and Adinortey et al. [25] in Ghana have strongly linked DM and diabetic retinopathy respectively to Hp polymorphism, raising current interests in research to elucidate the role of Hp phenotypes in DM and its complications. This study was thus conducted to provide data on Hp phenotypes and DM in Ghana with a view to determining whether the various Hp phenotypes have any association with major complications in diabetes.

In this study, diabetic patients recorded higher BMI which is consistent with the observation that higher BMI is a major risk factor for the development of DM [39]. A higher BMI recorded for T2DM patients in this study, is consistent with previous reports [40, 41]. The AST and ALT levels observed among participants were within reference ranges, and did not vary significantly among diabetics and non-diabetics, indicating that most participants had their normal liver functioning properly. Fasting blood sugar level for the non diabetic participants which was within the reference range, however, was significantly lower compared to the level for the T2DM participants. This confirms an earlier report [41].

There are many reports on Hp phenotypes, diabetes and its complications from all parts of the world [26, 30, 42–45]. However, there is controversy on whether there is any association between Hp phenotypes and predisposition to DM. For example, a report by Awadallah and Hamad [43] suggests that the prevalence of diabetes is independent of Hp phenotypes in Jordanians. Contrary to this, a population study by Stern et al. [42] indicates that diabetes is associated with the Hp 1–1 phenotypes. Results of the present investigation in Ghanaians support the position that diabetes is associated with Hp phenotypes. This finding confirms what has been earlier on reported by Quaye et al. [26]. However, in contrast to the observation by Stern et al.

[42], the results of this study indicate that there is a strong association between DM and the Hp 2–2 phenotype. The Hp 2–1 and Hp 1–1 registered equal frequencies followed by Hp 2–1 M and the least being Hp 0–0. (Hp 2–2 > [Hp 2–1 = Hp 1–1] > Hp 2–1 M > Hp 0–0). The reasons for the differences between our data for Ghanaians and that reported by Stern and colleagues are not immediately apparent. However, lifestyles are known to be risk factors for the development of DM [46] and this may override genetic predisposition between the two study populations.

Investigations have established a strong linkage between Hp phenotypes and certain diabetic complications. Our results indicate that complications among Ghanaian diabetics showed the least association with the Hp 1–1 phenotype. This finding is consistent with that of other investigators who have attributed the phenomenon to the protective function of Hp 1–1 [24, 26]. Diabetic complications were strongly associated with Hp 2–2 and Hp 2–1, Hp 1–1, Hp 2–1 M, in a decreasing order in this study. The reasons for this could be due to (i) differences in Hb binding capacities of the different Hp phenotypes [47]; (ii) the shape and size of the Hp phenotypes [17]; (iii) an increase in redox active iron in the plasma of humans with the Hp 2–2 phenotype [48]; (iv) the “clearance” of Hp–Hb complex in the blood circulation after intravascular haemolysis by CD163 which is less effective in Hp 2–2 individuals [23]; and (v) the better antioxidant effects of Hp 1–1 phenotypes on low-density lipoprotein (LDL) oxidation than Hp 2–2 [49, 50]. These may explain the susceptibility of Hp 2–2 individuals to complications such as coronary artery diseases (CAD), neuropathy, nephropathy and hypertension.

This study also showed an increased risk of complications for individuals with Hp 2–2 phenotype. For example, the odds ratio of having complications in a diabetic with an Hp 2–2 phenotype was 18.3 times greater than that for Hp 0–0. The strong association of all the complications recorded in this study with the Hp 2–2 phenotype may be related to the poor antioxidant activity associated with the Hp 2–2 phenotype compared to the other phenotypes. In this study, relatively lower complications were recorded among individuals who were of the Hp 2–1 M phenotype. This would suggest that the Hp 2–1 M phenotype is protective against diabetes and its complications. The reasons for this are not apparent. Further studies may be warranted in this direction.

Conclusion

In Ghana, DM and its complications are associated with the Hp 2–2 phenotype. Furthermore, the risk of developing diabetic complication is higher among diabetics who have

the Hp 2–2 phenotype. Hp 2–2 phenotype may therefore be a useful predictor for an individual to develop DM as well as complications associated with the disease. It would therefore be worthwhile for individuals to know their Hp phenotypes and therefore be able to adapt lifestyles that would delay the onset of complications.

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