Haptoglobin Polymorphism in Individuals with Type 2 Diabetic Microangiopathy

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Abstract

Background: Haptoglobin is an acute phase protein with antioxidant and immunomodulatory properties. Gene polymorphism may be a risk factor for diabetic vascular disease in Iranian population. Aims: The study investigates the existence or not of an association between haptoglobin genotypes and prevalence of diabetic microangiopathy in individuals with type 2 diabetic microangiopathy. Materials and Methods: We included 206 type 2 diabetic patients (>5 years duration) categorized into two groups according to the presence or absence of diabetic microvascular complications. The cases of interest were diabetic neuropathy, retinopathy, and nephropathy identified during clinical and/or laboratory examination. In addition, 114 age- and sex-matched individuals were selected to serve as a control group. Haptoglobin genotyping was done using an amplification gel electrophoresis. Results: The frequency of haptoglobin phenotype 2-1 in diabetic patients was 70/206 (33.9%) as compared with 54/114 (47.3%) in nondiabetics (P= 0.01). However, the frequency of haptoglobin phenotype 2-2 was greater in diabetics (126/114, 61.1%) than in those without diabetes (56/114, 49.1%; P = 0.02). Patients with diabetic microangiopathy, however, did not differ significantly between haptoglobin phenotype groups. Conclusions: Haptoglobin phenotype 2-2 is considered to be a major susceptibility gene for type 2 diabetic patients. Moreover, haptoglobin phenotype 2-1 may be good prognostic factors for the development of diabetes mellitus.

Keywords: Diabetes mellitus, Diabetic microvascular complications, Genetic polymorphisms, Haptoglobin genotypes

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Introduction

Haptoglobin (Hp) is a plasma a2-glycoprotein synthesized primarily by hepatocytes.[1] Synthesis is stimulated by infection or inflammation (the acute phase response). After release into the circulation it has a half-life of 2-4 days.[2] Hp binds oxygenated, free hemoglobin with a very high affinity of approximately 1 × 10^{-15} mol/l.[3] Free hemoglobin can be harmful to the body, promoting the accumulation of hydroxyl radicals via the Fenton reaction (H_{2}O_{2} + Fe^{2+} → Fe^{3+} + OH) + OH), resulting in oxidative tissue damage.[4] Once irreversibly bound to Hp, free hemoglobin loses its oxidizing ability.[4,5] The Hp-hemoglobin (Hp-Hb) complex is removed from the circulation by binding to a receptor (CD163) found on the cell surface of monocytes and macrophages.[6] Hp was first described as polymorphic by Smithies who used starch gel electrophoresis to separate the various types and the existence of two autosomal genes with incomplete penetrance was proposed by Smithies and Walker. In humans, two alleles (marked as 1 and 2) exist for the Hp gene. These give rise to three major phenotypes in man still commonly referred to as Hp1-1, Hp2-1, and Hp2-2.[7] It is an acute phase protein synthesized mainly in the liver and found at levels of 30-300 mg/dl...
in normal human serum, Hp serum levels are increased up to three- to eight-fold during the acute phase reaction and in response to injury.\textsuperscript{[8]} The hepatic synthesis of Hp is induced by interleukin-6 (IL-6), IL-1, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)). Hp is also expressed in specific nonhepatic cells, including adipocytes and lung cells, and its levels are increased after inflammation similar to that observed in hepatocytes.\textsuperscript{[9]} Moreover, 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.\textsuperscript{[10]}

Although diabetic disease duration is the main risk factor for the development of diabetic microvascular complications (MVCs); including nephropathy, neuropathy, and retinopathy; glycemic control; blood pressure; and other co-risk factors are modifier to disease duration. Hyperglycemia appears to be a necessary but not sufficient condition for the development of diabetic vascular disease.\textsuperscript{[11-15]} There exists a growing body of evidence that some genetic susceptibility had modifying effects on diabetic vascular disease develop.\textsuperscript{[16-18]} Considerable evidences have shown the importance of the generation of reactive oxygen species in the development of diabetic vascular complication.\textsuperscript{[19]}

There exists considerable \textit{in vitro} and \textit{in vivo} data supporting differences in the antioxidant, scavenging, and immunomodulatory properties between the different Hp types. Several researches demonstrated in prospective and cross-sectional population studies that a polymorphism in the Hp gene is an independent risk factor for diabetic vascular disease.\textsuperscript{[20,21]} The prevalence of diabetes mellitus (DM) in the Iranian population is estimated to be relatively high; around 8% in the population aged between 30 and 69 years.\textsuperscript{[22,23]} To our knowledge, there are no studies investigating the influence of Hp polymorphism on this subpopulation to date. In order to verify, if there is an association between Hp genotypes and the presence of diabetic related MVCs in general and each specific diabetic MVC in particular, we studied type 2 diabetic patients, with and without diabetic microangiopathy.

\section*{Materials and Methods}

Ethical certificate was obtained from the ethics committee of the molecular biology research center of Mazandaran University of Medical Sciences. This case-control study was conducted in Sari city where subjects in this study were Iranian people living in Mazandaran province, and all provided informed consent to participate.

The cases were 206 diabetic related MVCs/MVC under focus and the controls were type 2 DM without the respective MVCs/MVC under focus. The 206 subjects with type 2 DM had already been diagnosed and were outpatients of the Diabetes Research Center of Imam Hospital, Sari City, between December 2010 and August 2012 were recruited. Patients were also compared with a control group of 114 healthy individuals who had been studied from the same geographical region as a secondary target for comparison of the entire type 2 DM group with nondiabetics group. The MVCs of interest were diabetic neuropathy, retinopathy, and nephropathy identified during clinical and or laboratory examination. We estimated that a sample size of 218 cases and 116 controls would give 90\% power to detect an OR of 2.0 at 5\% level of significance given a prevalence of Hp in the community of 7-70\% and in individuals with diabetic MVCs of 6-10\%.\textsuperscript{[24]}

The inclusion criteria were defined as all diabetic patients with at least 5 years disease duration. Patients with pregnancy, heritable diseases (such as Biedle), using chronic drugs (such as corticosteroids), uveitis and retinal dystrophy, nondiabetic related renal diseases, nondiabetic neuropathy, and those aged 75 years or older were excluded from study.

Patients underwent a comprehensive clinical and ophthalmic evaluation. The following demographic, clinical, and laboratory information was documented for each subject: Mean age, sex, diabetes, age of onset, and duration, presence of hypertension (blood pressure was corrected according to sex and age, presence of chronic complications (neuropathy and proteinuria)), funduscropy, average glycosylated HbA1c and insulin usage. Blood pressure was measured at every clinic visit, with hypertension being defined as systolic pressure 140 mmHg or greater or diastolic pressure 90 mmHg or greater. The MVCs of interest were diabetic neuropathy, retinopathy, and nephropathy identified during clinical and or laboratory examination.

Fundus evaluation was performed by trained ophthalmologist and, in case of abnormalities, documented by fundus fluoresceine angiography. Retinopathy was defined by the presence of characteristic changes, including hemorrhages, exudates, laser marks, and fibrous proliferation, detected by ophthalmoscopy through dilated pupils. In the presence of diabetic retinopathy, it was classified as nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR).\textsuperscript{[25]}

All patients provided two separate 24 h urine collection samples. Urinary albumin concentration was measured by immunoturbidimetry with intra- and interassay coefficient of variance of 3.3 and 6.7\%, respectively. The lowest limit of detection was 2.5 mg/l.
Nephropathy was defined as an albumin excretion rate 30-300 mg/1/24 h as microalbuminuria and >300 mg/1/24 h as gross albuminuria in a timed urine collection after excluding urinary tract infection.[26]

Clinical evaluation for peripheral neuropathy includes examination for paresthesia, sensation loss, weakness, and decreased or absent deep tendon reflexes. The clinical staging of peripheral neuropathy was defined according to involvement of the 14 nerves (both median, ulnar, peroneal, posterior tibial motor nerves and both median, ulnar, sural sensory nerves) as follows: Stage 0 (no neuropathy); stage 1 (mild), one or two nerves affected; stage 2 (moderate), three or four nerves affected; stage 3 (severe), five or more nerves affected. Neurophysiologic studies with nerve conduction velocity (NCV) were performed with the Nicolet Viking IIe (Nicolet Biomedical, Madison, WI) machine. Motor conduction studies were measured with surface electrodes on the muscles and supramaximal stimulation of the corresponding nerves. Median and ulnar nerves were stimulated at the wrist and elbow, with motor action potential being recorded over the abductor pollicis brevis and abductor digiti minimi, respectively. Peroneal and tibial nerves were stimulated at the ankle and knee, with motor action potential being recorded over the extensor digitorum brevis and adductor hallucis, respectively. Sensory NCV were performed on the median, ulnar, and sural nerves; with orthodromic stimulation at finger II, finger V, and lateral malleolus, respectively. The sensory action potentials of the median and ulnar nerves were recorded at the wrist and at the lower leg for the sural nerve.[14]

The tests described in autonomic dysfunction are based on the responses of the heart rate and blood pressure to a variety of stimuli. The first three reflect cardiac parasympathetic integrity, while the other two start to give abnormal results with more severe sympathetic nerve damage. While each test may be used individually, we think that parasympathetic and sympathetic nerve tests should be performed when possible, hence giving complete information about the state of the autonomic nervous system. A test reflecting cardiac parasympathetic damage was performed with the patient lying quietly on a couch, while the heart rate is recorded continuously on an electrocardiograph. The patient is then asked to stand up unaided, and the point at starting to stand is marked on the electrocardiogram. The shortest R-R interval at or around the 15th beat and the longest R-R interval at around the 30th beat after starting to stand, is measured with a ruler. The characteristic heart rate response is expressed by the 30:15 ratios. Immediate heart rate response to standing (30:15 ratios) > 1 was normal and <1.00 was abnormal. The tests reflecting sympathetic damage was performed by measuring the patient’s blood pressure with a sphygmomanometer while he is lying down quietly and again when he stands up. The postural fall in blood pressure is taken as the difference between the systolic blood pressure in the lying and standing position. Blood pressure response to standing (fall in systolic blood pressure) <10 mmHg was normal and >30 mmHg was abnormal.[19]

The blood samples (5 ml) were drawn from the antecubital vein from all subjects into ethylenediaminetetraacetic acid (EDTA) and processed for HP genotyping. A fasting blood sample was collected aseptically from all subjects and was also collected on EDTA and analyzed within a few hours to estimate the percentage of glycosylated hemoglobin (HbA1c) using a fast ion exchange resin separation method,[27] serum samples were separated and immediately frozen until analyzed at central lab of medical school, Sari.

The genomic DNA was extracted from the remaining white blood cells using a Puregene DNA purification kit (Gentra Systems, Inc., Minneapolis, MN). The DNA samples were stored and shipped at 4°C. All kits and reagents were used according to their manufacturer’s instructions. HP genotyping were determined using conventional polymerase chain reaction (PCR). Amplification of the 1757-bp HP1 allele-specific fragment was performed in a volume of 20 μl, containing 4 μl of 5x Prime STAR Buffer (Mg2+ plus) (Takara, Shiga, Japan), 250 nmol/l each of primers A and B, and about 0.1-10 ng genomic DNA. The temperature profile was 35 cycles of denaturing at 98°C for 10 s and annealing and extension at 72°C for 2 min. The HP2 allele-specific fragment was amplified in a volume of 20 and 10 μl of Gotaq® Green Master mix (Promega, Madison, WI), 250 nmol/l each of primers C and D,[28] and about 0.1-10 ng genomic DNA. The temperature profile was 95°C for 1 min, followed by 35 cycles of denaturing at 96°C for 10 s, annealing and extension at 65°C for 30 s, and then final extension cycle at 72°C for 1 min. Primers for the conventional PCR were synthesized by Hokkaido System (Sapporo, Hokkaido, Japan). HP del zygosity was determined by duplex PCR using Hp-del-U, Hp-del-L, Hp-Ex1-U, and Hp-Ex1-L primers as described by Koda et al.[29] Resultant PCR products were size-fractionated by agarose gel electrophoresis.

Statistical analysis
Data are expressed as the mean ± standard deviation (SD), or proportions for categorical variables. HP genotypes variation was calculated using the chi-square test. Categorical valuables were compared using Fisher’s exact test. Nonparametric data were analyzed by the odds ratio (Or) test. Associations between the HP genotypes and DM, blood pressure, serum lipid values,
and diabetic microangiopathic change were evaluated. A \( P < 0.05 \) was considered to be statistically significant.

**Results**

The main clinical and biochemical data of the type 2 diabetic patients are shown in Table 1. This finding emphasizes that type 2 DM with MVCs where slightly older, have longer DM duration, and less controlled as can be evident by HbA1c though not statistically significant.

Data analyses after adjusting for age and gender showed that the statistical significance of diabetes duration in non-MVCs patients with DM to MVCs patients with DM was 0.001, and that the elevated HbA1c in non-MVCs patients with DM to MVCs patients with DM was 0.003 [Table 1]. These confirmed already known associations between MVCs and diabetes duration and elevated HbA1c levels in DM. There were no significant differences in HbA1c levels, hypertension, hypercholesterolemia, or duration of diabetes between patients with different Hp genotypes.

Among the diabetic participants; retinopathy, nephropathy, and neuropathy were found in 109 (52.9%), 57 (28.8%), and 103 (58.5%) individuals, respectively. The patients with diabetic neuropathy were either peripheral or autonomic in 103 (58.5%) and 61 (34.6%) individuals, respectively. In diabetic patients, 65.4% had at least one form of MVCs.

Table 2 shows the results of the Hp genotype distribution for diabetes patients and control subjects. The allele frequencies were also categorized according to the presence/absence of MVCs. The distribution of the three Hp genotypes was 4.4, 38.7, and 56.9% for Hp 1/1, 2/1, and 2/2, respectively. This result has shown that the Hp2-2 allele frequency is highly significant for type 2 diabetes versus control cases (61.1 vs. 49.1%), whereas Hp1-2 allele appears inversely (33.9 vs. 47.3%); \( P = 0.057 \).

| Characteristics                  | Control \((n=114)\) | DM with MVCs \((n=134)\) | DM without MVCs \((n=71)\) | \( P \)-value |
|----------------------------------|---------------------|--------------------------|---------------------------|--------------|
| Age (years)                      | 59.16±13.21         | 56.97±10.46              | 55.69±8.49                | 0.376        |
| Sex (M/F)                        | 45/69               | 42/92                    | 21/50                     | 0.46         |
| Diabetes duration (<10 years/>10 years) | —                  | 59/75                    | 49/21                     | 0.001        |
| HbA1c (<7.5%/>7.5%)              | —                   | 41/93                    | 35/35                     | 0.003        |
| Hypertension (no/yes)            | 51/58               | 98/36                    | 51/20                     | 0.870        |
| Hypercholesterolemia (no/yes)    | 23/40               | 56/26                    | 25/16                     | 0.432        |

DM: Diabetes mellitus; MVCs: Microvascular complications; M: Male; F: Female. Data are expressed as mean ± standard deviation (SD) or % (\( n \)). Data are in percentage (\( n \)) unless otherwise indicated.

| Groups / Variables | Hp1-1 \((n)\) | Hp2-1 \((n)\) | Hp2-2 \((n)\) | \( P \)-value |
|-------------------|---------------|---------------|---------------|--------------|
| DM without DR     | 4/97 (4.1%)   | 37/97 (38.1%) | 56/97 (57.7%) | 0.469        |
| vs. DM with DR    | 6/109 (5.5%)  | 33/109 (30.3%)| 70/109 (64.2%)|              |
| vs. DM without DN | 7/141 (4.9%)  | 49/141 (34.7%) | 85/141 (60.2%)| 1.000        |
| vs. DM with DN    | 3/57 (5.2%)   | 20/57 (35%)   | 34/57 (59.6%) |              |
| vs. DM without DNP| 4/73 (5.4%)   | 21/73 (28.7%) | 48/73 (65.7%) |              |
| vs. DM with DNP   | 5/103 (4.8%)  | 36/103 (34.9%)| 62/103 (60.1%) |              |
| vs. DM without DNa| 6/115 (5.2%)  | 33/115 (28.6%)| 76/115 (66%)  | 0.371        |
| vs. DM with DNa   | 3/61 (4.9%)   | 24/61 (39.3%)  | 34/61 (55.7%)  |              |
| vs. DM without MVCs| 3/71 (4.2%)  | 23/71 (32.3%)| 45/71 (63.3%)  | 0.937        |
| vs. DM with MVCs  | 7/134 (5.2%)  | 46/134 (34.3%)| 81/134 (60.4%) |              |
| Controls          | 4/114 (3.5%)  | 54/114 (47.3%)| 56/114 (49.1%) | 0.057        |
| vs. DM            | 10/206 (4.8%) | 70/206 (33.9%)| 126/206 (61.1%)|              |

DM: Diabetes mellitus; MVCs: Microvascular complications; DR: Diabetic retinopathy; DN: Diabetic nephropathy; DNP: Peripheral diabetic neuropathy; DNa: Autonomic diabetic neuropathy. Data are in percentage (\( n \)) unless otherwise indicated.
Predictive values of risk factors for MVCs using multiple logistic regression analysis are shown in Table 3. These data demonstrate that the relative risk of Hp 2-2 in diabetes was significantly greater than in nondiabetes, (confidence interval (CI) 0.386-0.973, OR = 0.613; \( P = 0.025 \)) and the relative risk of Hp 2-1 in diabetes was significantly lesser than in nondiabetes (CI 1.096-2.790, OR = 1.749; \( P = 0.013 \)).

**Discussion**

In this case-control of subjects with type 2 diabetes, we demonstrate no association between the Hp genotype and MVCs in general or any specific MVCs. Previous studies assessing the association between the Hp phenotype and the presence or incidence of renal disease have produced conflicting findings. In a small, cross-sectional study of subjects with type 1 or 2 diabetes, none of those with the Hp 1/1 phenotype exhibited signs of nephropathy (0/18) compared with 27% (10/37) of those with Hp 2/1 and 34% (19/55) of those with Hp 2/2 (\( P < 0.02 \)).[30,31] Similar results were reported from an Irish type 1 diabetes case-control study.[32] Conversely, separate study of American and Japanese individuals with type 2 diabetes did not observe an increased risk associated with the common Hp phenotype (\( P = 0.77 \) and 0.43, respectively).[33,34] Similarly, we were also not able to detect association for either microalbuminuria or macroalbuminuria incidence in our individuals with a long duration of type 2 diabetes, perhaps suggesting that at a more advanced stage of diabetes, early Hp-susceptible cases of nephropathy may have been excluded.

It has been shown that the Hp1-1 phenotype is associated with a reduced risk for the development of DR in a group of Nakhouli et al., DM1 patients.[35] The authors of this study suggested that the functional differences in antioxidant capacity between the phenotypes could be responsible for this association. On the other hand, Koda et al., investigating Japanese patients with DM2 did not find a protective effect of the HP1 allele. On the contrary, the Hp2-2 phenotype frequency was higher in patients without DR.[34] Similar results were reported from an Brazilian diabetes case-control study.[36] We could not find any significant differences in genotype frequencies for the DM patients with or without DR and all other types of MVCs analyzed here.

The only statistically significant results we obtained were in relation to the presence of diabetes that such differences may be important. This study has shown that the Hp2-2 allele is a high risk factor for type 2 diabetes in Iran whereas Hp1-2 allele appears protective. There are many reports on HP phenotypes, diabetes, and its complications from many parts of the world.[37-44] This was similar with Quaye et al., study that concluded that Hp2-2 allele was a risk factor for type 2 diabetes in Ghana, whereas the HP1-1 allele appeared protective.[37] However, this finding contrasts with previous observation in American-Indian studies where HP allele was a risk factor. These differences highlight role of genetic and ethnic variation determining susceptibility to type 2 diabetes.[40,41]

Glycosylated Hb is a more potent source of redox active iron and Hp2-2 has less ability to inhibit the release of such iron from glycosylated Hb.[42] The lack of simple associations between Hp type and disease may reflect the complex interactions between Hp and the immune system as well as antioxidant functions. There was no significant difference in between the Hp genotypes for any of the MVCs risk factors or DM characteristics as determined both by univariate analysis and by multinomial logistic regression analysis. The only statistically significant results we obtained were in relation to the already known associations between MVCs and diabetes duration and elevated HbA1c levels in DM, respectively. In conclusion, the lack of association between MVCs and polymorphism of the Hp gene indicates that Hp genotypes may not be genetic markers of predisposition to diabetic microangiopathy in this Iranian population. However, in respect to diabetes incidence, it would therefore be emphasizing that the difference in Hp distribution between type 2 diabetics and nondiabetics is very interesting.

**Table 3: Predictive values (odds ratio (OR)) of risk factors for MVCs using multiple logistic regression analysis**

| Factors for MVCs | Non-DM-DM P-values OR 95% CI | Non-DR-DR P-values OR 95% CI | Non-DN-DR P-values OR 95% CI | Non-DNp-DNp P-values OR 95% CI | Non-DNp-DNa P-values OR 95% CI | Non-MVCs -MVCs P-values OR 95% CI |
|-----------------|-----------------------------|------------------------------|----------------------------|-----------------------------|----------------------------|---------------------------------|
| Hpa-1          | 0.400 0.713 0.218-2.326     | 0.449 0.788 0.202-2.698      | 0.931 0.940 0.234-3.771     | 0.853 1.139 0.294-4.385     | 0.982 1.064 0.257-4.412       | 0.752 0.800 0.201-3.195        |
| Hpa-2          | 0.013 1.749 1.096-2.790      | 0.149 1.420 0.796-2.533      | 0.964 0.985 0.517-1.874     | 0.388 0.752 0.393-1.438     | 0.151 0.620 0.323-1.193        | 0.877 0.917 0.497-1.690        |
| Hpa-2          | 0.025 0.613 0.386-0.973      | 0.209 0.761 0.436-1.535      | 0.934 1.028 0.548-1.923     | 0.453 1.270 0.680-2.370     | 0.177 1.548 0.819-2.923        | 0.399 1.132 0.625-2.051        |

DM: Diabetes mellitus; MVCs: Microvascular complications; DR: Diabetic retinopathy; DN: Diabetic nephropathy; DOp: Peripheral diabetic neuropathy; DNa: Autonomic diabetic neuropathy; Hp: Haptoglobin; CI: Confidence interval.
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