Role of Hemochromatosis C282Y and H63D Mutations in *HFE* Gene in Development of Type 2 Diabetes and Diabetic Nephropathy

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OBJECTIVE — In patients with clinical hemochromatosis, the frequency of diabetes ranges from 20 to 50%, and the heterozygosity for the C282Y mutation in the *HFE* gene might be associated with an increased risk for diabetes. There are also some reports that suggest that iron overload might cause diabetic nephropathy.

RESEARCH DESIGN AND METHODS — We performed an association study to assess the role of the C282Y and H63D mutations in the *HFE* gene as a risk factor for type 2 diabetes and diabetic nephropathy. Altogether, 563 patients with type 2 diabetes were included in the study. In the analyzed group, 108 patients had overt proteinuria, 154 had microalbuminuria, and 301 had normoalbuminuria. Among the patients with normoalbuminuria, only those with known diabetes duration ≥10 years were considered normoalbuminuric (n = 162). A total of 196 unrelated healthy subjects were used as a control group. All subjects were genotyped for C282Y and H63D using the polymerase chain reaction–based protocol.

RESULTS — There was an increased frequency of 282Y allele carriers among patients with type 2 diabetes versus healthy control subjects (OR 5.3, 95% CI 1.6–17.3). We observed an increased frequency of the 63D allele carriers among patients with diabetic nephropathy (1.8, 1.2–2.8).

CONCLUSIONS — In conclusion, our study is the first to indicate that being a carrier of the H63D hemochromatosis mutation is a risk factor for nephropathy in type 2 diabetic patients. We also confirmed previous observations that the frequency of the 282Y mutation was higher in patients with type 2 diabetes than it was in the general population of healthy subjects.

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The C282Y mutation in the *HFE* (hemochromatosis) gene is the main one that causes hemochromatosis, and 83% of hemochromatosis patients are YY homozygotes (1). The second variant of the *HFE* gene, the H63D polymorphism, is not per se associated with hemochromatosis, but it acts synergistically with the C282Y mutation (1). In patients with clinical hemochromatosis, the frequency of diabetes ranges from 20 to 50% (2–5), and the heterozygosity for the C282Y mutation might be associated with an increased risk for diabetes (6). A large study of heterozygous subjects from hereditary hemochromatosis families showed that the relative risk for type 2 diabetes was marginally increased in men (6). Iron metabolism was abnormal in some subjects heterozygous for hereditary hemochromatosis, and the mean serum iron concentration and transferrin-saturation values were higher in heterozygotes than in normal subjects (7). Also, serum ferritin concentration was higher in heterozygotes than in normal subjects (7). Body iron stores have been shown to be associated with abnormal glucose tolerance and insulin resistance (8). Several studies that tried to confirm the hypothesis that heterozygosity for hereditary hemochromatosis-causing mutations could be a risk factor for diabetes produced somewhat controversial results. Kwan et al. (9) observed an increased frequency of C282Y mutations in patients with type 2 diabetes and concluded that the C282Y gene mutation was a potential genetic marker for type 2 diabetes. Fernandes-Real et al. (10) found that although the C282Y allele frequency was similar in patients with type 2 diabetes and control subjects, the H63D allele frequency was significantly increased in patients with type 2 diabetes. Several studies were unable to prove this association. In these studies, the frequency of C282Y and H63D mutations was not increased in patients with type 2 diabetes when compared with the general population (11–14). Therefore, we decided to examine whether the C282Y and H63D mutations were associated with type 2 diabetes in the Polish population.

Some reports suggest that iron overload might cause diabetic nephropathy. Nankivell et al. (15) observed an increased proximal tubular lysosomal iron concentration in subjects with diabetic nephropathy. Also, Loebstein et al. (16) observed an early development and an accelerated course of diabetic nephropathy in iron-loaded patients with β-thalassemia. Following this hypothesis, being a heterozygote for the hemochromatosis-causing mutation might be a risk factor for nephropathy in type 2 diabetes. Therefore, we performed an association analysis to assess the role of C282Y and

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Abbreviations: ACR, albumin/creatinine ratio; BP, blood pressure.
A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical characteristics of type 2 diabetic patients included in the study according to nephropathy status*

	Normo-albuminuria*	Micro-albuminuria	Proteinuria	Healthy control subjects
n	162	154	108	196
Male/Female	53/109	81/73	51/57	90/106
Age at examination (years)	64.3 ± 7.8†	62.0 ± 8.2†	62.5 ± 9.2	44.0 ± 7.8
Age at diagnosis of type 2 diabetes (years)	48.5 ± 9.4	49.7 ± 12.3	51.8 ± 9.8	—
Known duration of type 2 diabetes (years)	15.8 ± 5.5‡	11.2 ± 6.8‡	11.0 ± 7.0‡	—
BMI (kg/m^2)	29.3 ± 4.5	29.9 ± 4.3	29.5 ± 4.2	27.0 ± 4.2
HbA_{1c}(%)	8.9 ± 1.7	9.2 ± 1.7	8.9 ± 1.8	—
Hypertension (%)	51.8‡	62.8†	74.9†‡	—

Data are means ± SD. *Known diabetes duration ≥ 10 years; †$P < 0.05$; ‡ $P < 0.01$.

H63D mutations as a risk factor for diabetic nephropathy in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

— Each patient with type 2 diabetes (diagnosed according to the National Diabetes Data Group criteria) (17) attended the Outpatient Clinic for Diabetic Patients in Zabrze between 1 January 1998 and 31 December 1998; and if they were willing to participate in the study, they were screened for microalbuminuria in a random urine sample using the semiquantitative dipstick Micral II test (Boehringer Mannheim, Mannheim, Germany). The patients were screened for microalbuminuria, and the verification of renal status was ascertained by means of repeated measurements of albumin/creatinine ratio (ACR) (in random urine samples) and serum creatinine concentration and careful review of medical files. The albumin concentration in the urine was measured by an immunoturbidometric assay on a TurbiTimer with Albumin-Urine kits (Behring, Germany). Serum and urine creatinine concentrations were measured by spectrophotometry on a Kodak automated system. Normoalbuminuria was defined according to Warram et al. (18) as an ACR <1.9 mg/mmol for men and <2.8 for women. Overt proteinuria was defined as an ACR ≥28.2 for men and ≥40.2 for women (18). Microalbuminuria was defined as an ACR in the range between normoalbuminuria and overt proteinuria. Albuminuria and proteinuria were diagnosed based on the consensus of two of three determinations (the interval between the measurements was ≥2 weeks), and there were no data suggesting nondiabetic kidney disease in the medical files (data mostly suggested acute or chronic urinary tract infection, asymmetrical kidney size, an increase in serum creatinine level after ACE inhibition, rapid decline of renal function, or systemic symptoms of vasculitis). Altogether, 563 patients with type 2 diabetes were collected and genotyped for C282Y and H63D polymorphisms. In the analyzed study group, 108 patients had overt proteinuria, 154 had microalbuminuria, and 301 had normoalbuminuria. None of the analyzed patients were treated with kidney replacement therapy, and a persistent rise in serum creatinine ≥1.5 mg/dl (range 1.61–2.78) was observed in 10 patients with proteinuria. To eliminate the misclassification of patients with normoalbuminuria (because they could develop nephropathy later in the course of type 2 diabetes), we divided them into two subgroups. The first subgroup (n = 139) consisted of patients with duration of type 2 diabetes <10 years after diagnosis, and the second subgroup (n = 162) was composed of subjects with known diabetes duration ≥10 years. The second subgroup was considered normoalbuminuric in our analysis.

Patients were considered hypertensive if they were on antihypertensive medication or if the systolic or diastolic blood pressure (BP) taken at the time of microalbuminuria screening and during a subsequent visit to the clinic was ≥140 or 90 mmHg; BP was measured twice with a mercurium sphygmomanometer in the supine position after ≥10 min of rest, and the results were averaged. Of the 243 patients taking antihypertensive treatment, 162 were taking ACE inhibitors.

Additionally, we collected blood samples from 196 unrelated healthy subjects who worked at the local factory, and they were considered as a control group representing the local population. Diabetes was not diagnosed in any of them during the annual checkup.

All enrolled patients were Caucasian, and they gave written informed consent for participation in the study. The study protocol was approved by the Ethics Committee of the Silesian School of Medicine.

DNA analysis

Genomic DNA was extracted from whole blood using the MasterPureGenomic DNA Purification Kit (Epicentre Technologies, Madison, WI). All subjects were genotyped by polymerase chain reaction of the region that contained the C282Y mutation and digestion with the *Sna*BI restriction enzyme as previously described (19). To confirm the 282YY homozygote, a new antisense primer was used as described by Jeffrey et al. (20). The H63D polymorphism was genotyped using the primers reported by Feder et al. (1) and using the *Dpn*II restriction enzyme (19,21). Restriction enzyme digests were analyzed on 1.5% agarose gel.

Statistical analysis

Data for groups are presented as means ± SD. Mean and median values were compared using Student's t test and the Mann-Whitney rank-sum test as appropriate. A χ^2 test was used to compare the frequency of genotypes, an odds ratio was calculated, and 95% CI was provided (22). The Hardy-Weinberg equilibrium was examined by the goodness-of-fit χ^2 test with the continuity correction.

A multivariate logistic regression was performed for diabetic nephropathy. The H63D genotype, HbA_{1c} at the time of microalbuminuria screening, and diastolic or systolic BP (averaged results of systolic

Table 2—*Hemochromatosis C282Y and H63D polymorphisms in patients with type 2 diabetes and control subjects*

	Control subjects	Type 2 diabetic patients
C282Y		
All	196	563
CC	193 (98)	520 (92)
CY and YY	3 (2)	43 (7)*
OR (95% CI)†	Reference	5.3 (1.6–17.3)
Allele C‡	389	1,082
Allele Y	3	44
H63D		
All	196	563
HH	149 (76)	385 (68)
HD and DD	47 (24)	178 (32)
OR (95% CI)§	Reference	1.5 (1.0–2.1)
Allele H‖	342	928
Allele D	50	198

Data are *n* (%), unless otherwise indicated. *One subject was TT homozygote; †$\chi^2 = 8.48$, $P = 0.0036$, type 2 diabetic patients vs. control subjects; ‡$\chi^2 = 8.55$, $P = 0.0035$, allele C vs. allele Y; §$\chi^2 = 3.71$, $P = 0.0542$, type 2 diabetic patients vs. control subjects; ‖$\chi^2 = 4.61$, $P = 0.0317$, allele H vs. allele D.

or diastolic BP taken at the time of microalbuminuria screening and during subsequent visit) were included in the model.

RESULTS — The study patients with normoalbuminuria, microalbuminuria, and proteinuria did not differ with regard to age at diagnosis of type 2 diabetes or BMI (Table 1). Patients with normoalbuminuria had longer known duration of type 2 diabetes after diagnosis than subjects with microalbuminuria and proteinuria, but they were considered as normoalbuminuric with the assumption that the duration of type 2 diabetes after diagnosis should be ≥10 years to avoid misclassifications. As presented in Table 1, hypertension was more frequent in patients with microalbuminuria and proteinuria than in the subgroup with normoalbuminuria. HbA_{1c} levels did not differ among the subgroups.

As presented in table 2, there was a significantly higher frequency of the 282Y allele carriers (CY and YY genotypes) among patients with type 2 diabetes than healthy control subjects. Also, an increased frequency of 63D allele carriers was observed among type 2 diabetic patients, although it was only borderline significant. Only one patient with type 2 diabetes was found to be a YY homozygote. Nine patients with type 2 diabetes and none of the healthy control subjects were compound C282Y/H63D heterozygotes. The results were similar when allele frequencies were analyzed.

Table 3 presents the comparison of the C282Y polymorphism frequency between type 2 diabetic patients with diabetic nephropathy (i.e., microalbuminuria and proteinuria) and those who were normoalbuminuric after ≥10 years of known type 2 diabetes duration. The type 2 diabetic patients with nephropathy had a higher frequency of C282Y than patients with normoalbuminuria; however, because of the low frequency of the 282Y heterozygotes, significance was not reached. Table 3 also shows the distribution of the H63D polymorphism among type 2 diabetic patients with and without diabetic nephropathy. There was a significantly higher frequency of the 63D allele carriers (HD and DD genotypes) among those with diabetic nephropathy. Three patients with normoalbuminuria and four patients with nephropathy were compound C282Y/H63D heterozygotes. The distribution of C282Y and H63D genotypes was not distorted from the Hardy-Weinberg equilibrium in all analyzed groups of patients.

Table 4 shows multivariate logistic regression analysis for diabetic nephropathy. In addition to the H63D genotype, other variables such as HbA_{1c} and systolic or diastolic BP, were entered into the model. When HbA_{1c} and systolic (OR 1.91, 95% CI 1.22–2.99) or diastolic (1.78, 1.14–2.77) BP was acccounted for, the presence of the HD or DD genotypes was a risk factor for the development of diabetic nephropathy. Because of a strong correlation between systolic and diastolic BP, they were not entered together into one model. Table 4 presents the results of two models, the first with systolic and the second with diastolic BP.

We did not observe a dose relationship in albuminuria among the H63D genotypes (data not shown).

Table 3—*Hemochromatosis 282Y and H63D polymorphisms in patients with type 2 diabetes according to renal status*

	Normoalbuminuria*	Nephropathy†
282Y		
All	162	262
CC	152 (94)	237 (90)
CY and YY	10 (6)	25 (10)
OR (95% CI)‡	Reference	1.6 (0.7–3.4)
Allele C§	313	499
Allele Y	11	25
H63D		
All	162	262
HH	122 (75)	165 (63)
HD and DD	40 (25)	97 (37)
OR (95% CI)‖	Reference	1.8 (1.2–2.8)
Allele H¶	278	417
Allele D	46	107

Data are *n* (%), unless otherwise indicated. *Only patients with duration of type 2 diabetes ≥10 years after diagnosis; †nephropathy means microalbuminuria or proteinuria; ‡$\chi^2 = 1.09$, $P = 0.2968$, normoalbuminuria vs. nephropathy; §$\chi^2 = 0.62$, $P = 0.4293$, allele C vs. allele Y; ‖$\chi^2 = 6.4$, $P = 0.011$, normoalbuminuria vs. nephropathy; ¶$\chi^2 = 4.83$, $P = 0.0280$, allele C vs. allele Y.

CONCLUSIONS — Our study found that the hemochromatosis-causing mutations C282Y and H63D played a role as risk factors for type 2 diabetes and determined the genetic susceptibility to diabetic nephropathy. The C282Y mutation was more frequent in type 2 diabetic patients than in healthy control subjects. Only 1 of 563 type 2 diabetic patients analyzed was a 282YY homozygote, but there were nine compound C282Y/H63D heterozygotes among type 2 diabetic patients. Neither 282YY homozygote nor compound C282Y/H63D heterozygote was observed among 196 healthy control subjects. Additionally, an increased frequency of H63D mutation was observed in type 2 diabetic patients with microalbuminuria and proteinuria versus those who stayed normoalbuminuric after ≥10 years of known diabetes duration. Our study is the first that assessed the role of the hemochromatosis mutations in the susceptibility to diabetic nephropathy in type 2 diabetes.

The results concerning the risk of type 2 diabetes are consistent with some previous studies (9–10) but contradictory to others, which do not observe any association between the hemochromatosis mutations and type 2 diabetes (11–14). Previous studies that assessed whether heterozygosity for the hemochromatosis is associated with type 2 diabetes have produced controversial results. These discrepancies may have been caused by a different frequency of hemochromatosis mutations in distinct ethnic groups, and

Table 4—*Multivariate logistic regression analysis for diabetic nephropathy*

Risk factor	Unit	Odds ratio	95% CI	*P*
Model 1				
H63D genotype	HD or DD	1.91	1.22–2.99	0.0052
HbA_{1c}	1%	1.02	0.90–1.14	NS
Systolic BP	10 mmHg	1.20	1.09–1.33	0.0004
Model 2				
H63D genotype	HD or DD	1.78	1.14–2.77	0.0113
HbA_{1c}	1%	1.01	0.90–1.13	NS
Diastolic BP	10 mmHg	1.22	1.03–1.44	0.0219

In addition to the H63D genotype, HbA_{1c} and systolic or diastolic BP were entered into the model.

the observed association may be specific only for some populations.

It appears plausible that even a lesser accumulation of iron, as observed in heterozygotes, can alter the glucose and insulin homeostasis of the body. There are several potential explanations why an increased accumulation of iron may increase the risk of type 2 diabetes. An increased accumulation of iron could affect insulin synthesis and secretion in the pancreas (23–24). Furthermore, increased body iron could enhance oxidation of free fatty acids through accelerated production of free radicals (25). Increased free fatty acid oxidation diminishes glucose utilization in muscle tissue and increases gluconeogenesis in the liver, leading to increased insulin resistance (23–25). In addition, accumulating iron could interfere with the insulin-extracting capacity of the liver (26). Studies in noncirrhotic hemochromatosis and in hypertransfused patients with β-thalassemia support the evidence that accumulation of iron leads to development of insulin resistance (26–27).

The study of Tuomainen et al. (8) showed an independent positive association between serum ferritin concentrations and markers of glucose homeostasis. Also, Salonen et al. (28) found that fasting concentrations of serum insulin and blood glucose were increased in men with high serum concentration of ferritin, an indication of increased stores of iron. Men with high stores of iron were more likely to develop diabetes than men with lower stores of iron (28). This study supports the theory that increased iron stores, even in the range not considered to be associated with hemochromatosis, contribute to the development of type 2 diabetes (28). Among the large number of environmental and genetic factors contributing to the development of diabetes, excess body iron appears to be a potential new candidate (8).

There were only a few studies dealing with the problem of increased iron accumulation and the risk of diabetic nephropathy. They suggested that accumulation of iron might cause kidney damage during the course of diabetes. Loebstein et al. (16) hypothesized that there might be an increased incidence and an accelerated course of diabetic nephropathy in patients with diabetes that is secondary to transfusional hemochromatosis. Diabetic nephropathy occurred earlier in diabetic patients whose diabetes was secondary to transfusional hemochromatosis than in the general diabetic population. In other reports, microalbuminuria was not detected in nondiabetic, iron-overloaded thalassemia patients (29). Although there is no evidence that thalassemia directly affects the development of nephropathy, excess iron may be a risk factor for nephropathy in the course of diabetes. It was postulated that these findings might be attributed to high oxidative stress in these patients, which is secondary to iron-derived free radicals and to the patients' diminished antioxidant reserves (16). Furthermore, Nankivell et al. (15) observed an increased proximal tubular lysosomal iron concentration and increased numbers of iron-containing lysosomes in patients with diabetic nephropathy. They postulated that iron played a role in causing tubular damage in diabetic patients (15).

It must be acknowledged that a case control study design may be subject to error because of unrecognized population stratification. Positive association observed in this model may be present even in the lack of genetic linkage if patients carrying the hemochromatosis mutations represent a subpopulation characterized by an increased risk of type 2 diabetes or diabetic nephropathy. Although all subjects in our study originated from Poland, we were not able to exclude population stratification. Therefore, the positive results of the study have a preliminary nature and need to be confirmed in other populations and in a family-based study design.

Furthermore, a prospective study needs to be performed to prove the role of altered iron metabolism in susceptibility to diabetic nephropathy. In our cross-sectional study design, it is only an inference from our genotypical data.

In conclusion, our study is the first to indicate that being a carrier of the hemochromatosis mutation is a risk factor for nephropathy in type 2 diabetes. We also confirmed previous observations that the frequencies of 282Y and 63H mutations were higher in patients with type 2 diabetes than in the general population of healthy subjects. However, because of the limitations of a case control study, the results should be confirmed in other populations and in a family-based study design.

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