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ORIGINAL ARTICLE

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A hemochromatosis-causing mutation C282Y is a risk factor for proliferative diabetic retinopathy in Caucasians with type 2 diabetes

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Abstract Iron metabolism might be involved in the pathogenesis of type 2 diabetes and in the pathogenesis of diabetic retinopathy. C282Y and H63D mutations in the hemochromatosis (HFE) gene are associated with increased serum iron levels and consequently with hereditary hemochromatosis. In the present study, we searched for a relationship between C282Y and H63D gene mutations and the development of proliferative diabetic retinopathy in Caucasians with type 2 diabetes. For this purpose, 90 subjects with type 2 diabetes with proliferative diabetic retinopathy (PDR) were compared to 133 diabetic subjects without PDR. There was a significantly higher frequency of the C282Y heterozygotes in patients with PDR compared to subjects without it (OR = 3.0, 95% CI = 1.2–8.0; $p = 0.02$), whereas no association was demonstrated between PDR and H63D genotypes (OR = 1.1, 95% CI = 0.6–2.2; $p = 0.7$). Logistic regression analysis revealed that the C282Y mutation was a significant independent risk factor for the development of PDR (OR = 6.1, 95% CI = 1.2–30.5; $p = 0.027$). These data suggest that heterozygosity for C282Y might be a novel risk factor for PDR in Caucasians with type 2 diabetes.

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Introduction

Diabetic retinopathy remains a leading cause of loss of vision in Europe and North America in working population. Despite improvements in metabolic control, proliferative diabetic retinopathy (PDR) continues to occur and affects approximately 10–20 % of subjects with type 2 diabetes. PDR represents a serious long-term diabetic complication with a substantial impact on the quality of life of diabetic patients (Klein et al. 1984).

Although several risk factors for progression of diabetic retinopathy to PDR have been identified (Porta et al. 2001, Keen et al. 2001), the underlying pathogenesis is still not known. PDR is characterized by active angiogenesis and the formation of fibrovascular tissue at the vitreoretinal interface. This process requires a local production of cell-derived angiogenic factors and synthesis of extracellular matrix components necessary for the anchorage of migrating endothelium.

The association between diabetic retinopathy and idiopathic hemochromatosis was reported in 1978 (Walsh and Malins 1978). Hereditary hemochromatosis is a late-onset, autosomal recessive disorder leading to a chronic iron overload syndrome. Hereditary hemochromatosis is the most common single gene disorder in northern Europeans that occurs with a frequency of approximately 0.5%. In 1996, HFE, a gene for hereditary hemochromatosis, was cloned; the HFE gene is normally expressed in crypt enterocytes of the duodenum (Parkkila et al., 1997). Two of the 37 allelic variants of the HFE gene described to date (C282Y and H63D) are significantly correlated with hereditary hemochromatosis (Hanson et al. 2001). In the hereditary hemochromatosis, there is a primary defect in the HFE gene leading to increased intestinal absorption of dietary iron and net iron accumulation (Hanson et al. 2001).

Iron metabolism has been recently associated with angiogenesis and extracellular matrix remodeling and fibrosis (Gardi et al. 2002, Parkes et al. 2000, Simonart et al. 2001). Moreover, emerging scientific evidence has disclosed bidirectional influences between iron metabolism and type 2 diabetes (Fernandez-Real et al. 2002). We therefore hypothesized that C282Y and H63D mutations could be associated with an increased risk of PDR progression via increased iron concentrations.

To test this hypothesis, we typed for C282Y and H63D gene mutations a group of 223 patients with type 2 diabetes divided in two groups according to the presence or absence of PDR.

Patients and methods

In this cross-sectional, case-control study, 223 unrelated Caucasian subjects of Slovenian origin with type 2 diabetes with a defined ophthalmologic status were enrolled. For the diagnosis of diabetes mellitus, the standard WHO (1985) criteria were used. Subjects were recruited from the Eye Clinic and from the Diabetic Outpatient Clinic of the University Medical Centre Ljubljana. Staging of diabetic retinopathy was performed by a senior ophthalmologist (MP) after pupil dilatation (tropicamide and phenylephrine 2.5%) using direct ophthalmoscopy and was electronically documented with a 50°-angle fundus camera (Topcon-TRC 40-IX; Tokyo, Japan). The study group consisted of 90 patients with PDR (new vessel formation or fibrous proliferation). The control group consisted of 133 subjects without PDR: 80 subjects with type 2 diabetes of more than 10 years' duration having no clinical signs of diabetic retinopathy, and 53 subjects with non-PDR (microaneurysms, retinal hemorrhages, hard exudates). To avoid the confounding effect of impaired kidney function, the patients with overt nephropathy were not enrolled. After informed consent was obtained, a detailed interview was made.

Arterial hypertension was defined as systolic blood pressure higher than 140 mmHg, diastolic blood pressure higher than 90 mm Hg, or both, at repeated measurements, or current use of antihypertensive agents for the confirmed diagnosis of arterial hypertension. Total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL), and triglycerides were determined using standard biochemical methods. Patient and control subject characteristics are listed in Table 1; the groups were matched for most clinical parameters except for age and insulin treatment. The study was approved by the National Medical Ethics Committee.

Analysis of HFE gene mutation was performed using standard PCR with the primers described by Feder et al. (1996). After amplification, aliquots of the PCR products were digested with *RsaI* (C282Y) and *MboI* (H63D) restriction enzymes. The resulting fragments were run on a 3% Nusieve 3:1 agarose gel and observed under UV illumination after ethidium bromide staining. Genotyping was done by two researchers (DP, BP) who were blinded to patients' retinopathy status. Differences in mean values were assessed by unpaired Student's t test and presented as means ± SD. Chi-square test was used to compare discrete variables. Logistic regression analysis was performed to assess the independent role of C282 mutation and other variables, including age, the age at onset of diabetes, gender, $HbA_{1c,}$ systolic and diastolic blood pressure, and insulin therapy (categorical variable: insulin therapy or no therapy). Statistical significance was set at $p < 0.05$. Statistical analysis was performed using the SPSS program for Windows version 11 (SPSS Inc. Il, USA).

Results

Clinical characteristics of the two groups are summarized in Table 1. Frequency of the genotypes of the hemochromatosis C282Y and H63D mutations are shown in Table 2. Genotype distribution of the hemochromatosis C282Y and H63D mutations in subjects with and without PDR were compatible with Hardy-Weinberg expectations (C282Y: PDR $\chi^2 = 0.545$, $p = 0.46$; without PDR $\chi^2 = 30.097$, $p = 0.755$; H63D: PDR $\chi^2 = 0.115$, $p = 0.735$; without PDR $\chi^2 = 1.01$, $p = 0.31$).

There was a significantly higher frequency of the C282Y mutation in subjects with compared to without PDR (OR = 3.0, 95% CI = 1.2–8.0; $p = 0.02$). There was no C282Y homozygote among 223 type 2 diabetic patients. In contrast, the H63D genotype was not significantly associated with PDR ($p = 0.7$). Additionally, there was one (1.1%) compound heterozygote of C282Y and H63D in patients with PDR and one (0.8%) compound

Table 1 Clinical characteristics of type 2 diabetes patients. *PDR* proliferative diabetic retinopathy

Characteristics	Subjects with PDR	Subjects without PDR	*P* value
Number	90	133	
Age (years)	64.1 ± 9.1	69.1 ± 9.2	< 0.001
Male (%)	45 (50)	60 (45)	n. s.
Duration of diabetes (years)	18.8 ± 8.8	17.8 ± 7.9	n. s.
Insulin therapy	64 (71.1)	64 (48.1)	< 0.001
Age at onset of diabetes	45.2 ± 10.9	51.5 ± 10.3	< 0.001
HbA_{1c} (%)	8.2 ± 1.8	8.3 ± 1.7	n. s.
Systolic blood pressure (mmHg)	147 ± 26	146 ± 21	n. s.
Diastolic blood pressure (mmHg)	88 ± 11	85 ± 8	0.02
BMI (kg/m^2)	28.0 ± 4.4	27.9 ± 4.6	n. s.
History of hypertension (%)	65 (70.4)	95 (71.4)	n. s.
Smokers (%)	13 (14.4)	20 (15.0)	n. s.
Total cholesterol (mmol/l)	5.3 ± 1.3	5.4 ± 1.2	n. s.
HDL cholesterol (mmol/l)	1.2 ± 0.4	1.2 ± 0.3	n. s.
LDL cholesterol (mmol/l)	3.2 ± 1.10	3.1 ± 0.9	n. s.
Triglycerides (mmol/l)	2.4 ± 1.6	2.5 ± 1.9	n. s.

Table 2 Genotype distributions of C282Y and H63D mutations in patients with and without proliferative diabetic retinopathy (PDR)

Mutation/genotype	Subjects with PDR	Subjects without PDR	OR (95% CI)	*P* value
C282Y				
YY homozygote	0 (0)	0 (0)		
CY heterozygote	13 (14.4)	7 (5.3)	3.0 (1.2–8.0)[1]	0.02[2]
CC homozygote	77 (85.6)	126 (94.7)		
H63D				
DD	1 (1.1)	3 (2.3)	1.1 (0.6–2.2)[3]	0.7[4]
HD	20 (22.2)	25 (18.8)		
HH	69 (76.7)	105 (78.9)		

[1]Odds ratio (95% confidence interval) for dominant model (YY plus CY versus CC)
[2]*P* value for dominant model (YY plus CY versus CC)
[3]*P* value for dominant model (DD plus HD versus HH)
[4]Odds ratio (95% confidence interval) for dominant model (DD plus HD versus HH)

Table 3 Logistic regression analysis for the association with retinopathy among type 2 diabetes patients

Risk factor	OR (95% CI)	*P* value
C282Y	6.1 (1.2–30.5)	0.027
Age	1.05 (0.99–1.11)	0.064
Insulin therapy	2.8 (1.3–6.1)	0.01
Age at onset of diabetes	1.03 (0.98–1.08)	0.21
HbA_{1c} (%)	1.2 (0.94–1.48)	0.16
Systolic blood pressure	1.01 (0.99–1.03)	0.18
Diastolic blood pressure	0.91 (0.85–0.96)	0.001

heterozygote of C282Y and H63D in subjects without PDR (OR 0.8, 95% CI 0.04–11.1; $p = 0.8$).

Logistic regression analysis (Table 3) showed that in addition to diastolic blood pressure and therapy with insulin, heterozygotes for C282Y mutation had a significantly increased risk of PDR (OR = 6.1, 95% CI = 1.2–30.5; $p = 0.027$), suggesting that the C282Y mutation in the HFE gene is an independent risk factor for PDR.

Discussion

Carriers of heterozygous hemochromatosis are usually asymptomatic, although the levels of transferrin saturation, serum iron, and serum ferritin differ significantly from those in healthy subjects (Bulaj et al. 1996). Heterozygosity for the C382Y mutation was associated with a fourfold risk of iron overload (Hanson et al. 2001). Heterozygosity for hemochromatosis-causing mutation C282Y may therefore be a common genetic marker of lifelong moderate iron overload, whereas serum iron and ferritin concentrations are influenced by short-term effects, such as inflammation, iron intake, alcohol intake, blood loss, diurnal variation, and other illnesses (infections, neoplasia) (Baynes 1996, Bulaj et al. 1996, Cook et al. 1976). Several studies investigating the association of C282Y heterozygosity with morbidity have given conflicting results, as is exemplified by diabetes, cardiovascular diseases, extrahepatic cancers, and alcoholic liver disease (Fuchs et al. 2002). On the other hand, such association seems unambiguous in the case of sporadic porphyria cutanea tarda (O'Reilly et al. 1997).

It has been recently reported that in the liver and heart increased iron concentrations could increase collagen synthesis and thus fibrosis as well as matrix metalloprotease-2 activity involved in extracellular matrix remodeling (Gardi et al. 2002, Parkes et al. 2000). Moreover, it has been suggested that iron may give a survival advantage to endothelial cells by increasing Bcl-2 expression (Simonart et al. 2001). Both active angiogenesis and the formation of fibrovascular tissue at the vitreoretinal interface are key processes in the pathogenesis of PDR.

Whereas this is the first report of a possible association between the hemochromatosis mutation and complications of diabetic retinopathy, such an association has been reported previously in the case of another microvascular complication of diabetes—diabetic nephropathy. In 1994, it was reported (Nankivell et al. 1994) that iron overload might cause diabetic nephropathy through accumulation in tubular lysosomes. Moreover, Moczulski et al. (2001) have recently reported that hemochromatosis-causing mutation H63D, but not C282Y, predisposes to diabetic nephropathy. It has been demonstrated that the C282 mutation alters the HFE protein structure and $beta_2$-microglobulin association or cell-surface expression, whereas the H63D mutation, in contrast, does not appear to prevent $beta_2$-microglobulin association or cell-surface expression, indicating that the C282 mutation results in a greater loss of protein function than does H63D (Feder et al. 1997, Lebron et al. 1998).

Moreover, our study demonstrates that insulin therapy, independently of the C282Y mutation, was statistically significantly associated with PDR. This finding suggests the existence of other factors, such as differences in the ability of insulin secretion, differences in the frequency of episodes of hypoglycemia, or adverse events associated with insulin therapy (hypoglycemia, worsening diabetic retinopathy if HbA_{1c} decreases rapidly) (DeWitt and Hirsch 2003, Dahl-Jorgensen et al. 1985).

It should also be acknowledged that a case-control study design might be subject to error because of unrecognized population stratification, therefore a positive association may be present even when genetic linkage is lacking. Positive results of our association study (the C282 mutation with PDR) may be spurious due to type I error (false positive association study).

In conclusion we suggest that the C282Y mutation might be a novel risk factor for PDR in patients with type 2 diabetes.

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