Genetic Polymorphisms in Genes Encoding Antioxidant Enzymes Are Associated With Diabetic Retinopathy in Type 1 Diabetes

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OBJECTIVE — Oxidative stress plays an important role in the development of microangiopathic complications in type 1 diabetes. We investigated polymorphic markers in genes encoding enzymes regulating production of reactive oxygen species in association with diabetic retinopathy or diabetic nephropathy.

RESEARCH DESIGN AND METHODS — A total of 124 patients with type 1 diabetes were investigated in this case-control study. All subjects were matched for sex, age, and duration of diabetes. Genotyping was conducted using real-time PCR for p.Val16Ala polymorphism in the *MnSOD* gene and c.C−262T in the promoter region of the *CAT* gene. Multiplex PCR method was used for determination of *GSTM1* and *GSTT1* polymorphic deletions. Fluorescence-labeled PCR amplicons and fragment analysis was used for assessing the number of pentanucleotide (CCTTT)n repeats in inducible nitric oxide synthase.

RESULTS — A positive association of *MnSOD* genotype Val/Val (odds ratio [OR] 2.49, 95% CI 1.00–6.16, $P = 0.045$) and *GSTM1–1* genotype (2.63, 1.07–6.47, $P = 0.031$) with diabetic retinopathy but not with diabetic nephropathy was demonstrated. Additionally, the combination of the two genotypes conveyed an even higher risk (4.24, 1.37–13.40, $P = 0.009$). No other investigated genetic polymorphisms were associated with either diabetic retinopathy or diabetic nephropathy.

CONCLUSIONS — Selected polymorphisms in genes encoding MnSOD and GSTM1 could be added to a panel of genetic markers for identification of individuals with type 1 diabetes at an increased risk for developing diabetic retinopathy.

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S everal studies have suggested that reactive oxygen species (ROS) are implicated in the etiology of type 1 diabetes (1) as well as in the development of severe microangiopathic complications such as diabetic retinopathy and diabetic nephropathy (2). Chronic extracellular hyperglycemia in diabetes stimulates ROS production and increases oxidative stress (3). The oxidation of high levels of glucose inside diabetic cells produces more electron donors (NADH and $FADH_2$) and increases the electron transfer, thereby generating superoxide (4).

Excess generation of ROS such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) and reactive nitrogen species such as nitric oxide oxidize target cellular proteins, nucleic acids, or membrane lipids and damage their cellular structure and function (4). Hyperglycemia stimulates the expression of inducible nitric oxide synthase (iNOS) and increases production of nitric oxide,

an intracellular second messenger (2). Increased nitric oxide generation accompanied by the superoxide overproduction favors the formation of peroxynitrite, a highly reactive oxidant (5). Evidence suggests that ROS also regulate the expression of genes encoding for proteins involved in inflammation, immune response, and cell death (6).

Antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and catalase (CAT) directly eliminate ROS, while glutathione-S-transferases (GSTs) detoxify cytotoxic secondary metabolites of ROS. Together they represent a protective mechanism against the damage caused by the oxidative stress. Most of the enzymes involved in the defense against oxidative stress are polymorphic.

Associations between *MnSOD* Val16Ala single nucleotide polymorphism (SNP) and diabetic nephropathy (7) or diabetic retinopathy in type 2 diabetes (8) and polyneuropathy in type 1 diabetes (9) emphasize the importance of polymorphism in these genes. Individuals carrying the homozygous TT or heterozygous CT genotype in the promoter region of the *CAT* gene have significantly higher enzyme activity, which offers a degree of protection against development of polyneuropathy (10). Human *GSTM1* and *GSTT1* polymorphic deletion are associated with the age of onset of type 1 diabetes (11). Variable expression of the human *iNOS* gene polymorphic pentanucleotide (CCTTT)n repeat is associated with different autoimmune diseases including type 1 diabetes (12). All described polymorphisms could add to the interindividual variability in patients with type 1 diabetes for the development of microangiopathic complications.

The aim of our study was to evaluate the association of polymorphic markers in genes encoding antioxidant enzymes, which share a common detoxification pathway (MnSOD, CAT, GSTM1, and GSTT1) or regulate ROS production (iNOS) with diabetic retinopathy or diabetic nephropathy in a cohort of patients with type 1 diabetes.

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RESEARCH DESIGN AND METHODS

Individuals with type 1 diabetes were recruited from the national register of childhood-onset type 1 diabetes. Diagnosis of type 1 diabetes was based on the World Health Organization/American Diabetes Association definition of diabetes (13). All patients were screened prospectively for the presence of diabetic retinopathy by annual dilated eye examination and fundus photography performed by an ophthalmologist experienced in diagnosing the presence of diabetic retinopathy (14). The presence of diabetic nephropathy was screened annually by assessing microalbuminuria in a random urine specimen with a dipstick (Micral-test; Roche Diagnostics, Mannheim, Germany) and confirmed if positive by the determination of the urine albumin-to-creatinine ratio from a second morning urine specimen. A value of >100 mg/g repeated on at least two of three measurements was considered diagnostic for diabetic nephropathy (15).

The study population consisted of 124 unrelated individuals with type 1 diabetes (70 male and 52 female patients, median age 27.12 years). All patients were treated with basal-bolus insulin regimen with at least four daily injections or an insulin pump. They were divided into two groups: patients with diabetic retinopathy or diabetic nephropathy ($n = 62$, case subjects) and patients without complications after at least 11 years of duration of type 1 diabetes ($n = 62$, control subjects). Both groups were matched by sex, age, and duration of diabetes. The clinical characteristics of participating patients are shown in Table 1. Mean A1C was calculated from all available values over the observation period with typically three to four values per patient annually. All of the participating patients were of Caucasian origin and had type 1 diabetes onset before 16 years of age. Samples for DNA extraction were obtained during a routine annual screening for diabetes-related conditions. This population-based case-control study was approved by the National Medical Ethics Committee. All participating patients gave their written informed consent.

Materials and methods

Genomic DNA was extracted from peripheral blood (10 ml) using a FlexiGene DNA isolation kit (Qiagene, Hilden, Germany) according to the recommended protocol.

Table 1—*Characteristics of patients with type 1 diabetes with chronic complications (diabetic retinopathy or diabetic nephropathy case subjects) and without chronic complications (control subjects)*

	Control subjects	Case subjects	*P*
n	62	62	
Age (years)	26.8 ± 5.5	27.4 ± 5.8	0.072
Sex (M/F)	35/27	35/27	—
Age at onset (years)	8.5 ± 4.4	7.3 ± 3.4	0.257
Duration of diabetes (years)	17.9 ± 5.6	19.3 ± 5.8	0.087
Mean A1C	8.1 ± 1.1	8.2 ± 1.0	0.748

Data are means ± SD unless otherwise indicated. Patient groups were matched by sex, age, age at onset, and duration of diabetes. *P* values are a result of the *t* test for comparison of continuous parameters between both groups.

MnSOD and CAT genotyping

For *MnSOD* Val16Ala polymorphism and *CAT* C-262T promoter polymorphism, real-time PCR genotyping was conducted on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). TaqMan SNP Genotyping Assays were used to determine the regions encompassing polymorphic sites. For detection of Val16Ala (ref. SNP ID: rs4880), assay ID:C_8709053_10 was used, and for *CAT* C-262T (ref. SNP ID: rs1001179), assay ID:C_11468116_10 was used. The reaction mixture (5 μl) contained 0.125 μl TaqMan SNP genotyping assay, 2.5 μl TaqMan Universal PCR Master Mix, and 100 ng extracted genomic DNA. A thermo-cycling program was performed according to the manufacturer's recommendations.

GSTT1 and GSTM1 genotyping

GSTT1 and *GSTM1* polymorphic deletions were identified using a multiplex PCR-based method with three sets of primers. For *GSTT1* polymorphism, the sequences of the forward and reverse primers were 5′-ATG TGA CCC TGC AGT TGC-3′ and 5′-GAG ATG TGA GGA CCA GTA AGG AA-3′. For GSTM1 polymorphic deletion, the forward and reverse primers were 5′-GCT TCA CGT GTT ATG GAG GTT-3′ and 5′-GAG ATG AAG TCC TTC AGA-3′. A third set of primers were 5′-GAA GAG CCA AGG ACA GGT AC-3′ and 5′-CAA CTT CAT CCA CGT TCA CC-3′ used for co-amplification of the human β-globin gene, an internal control of positive amplification in the case of the presence of deletion in both genes. Such genotyping approach did not allow for detecting heterozygous carriers of *GSTM1* or *GSTT1* deletion; hence, the *GSTM1-0* or *GSTT1-0* genotype group included only patients homozygous for *GSTM1* or *GSTT1* deletion. The *GSTM1-1* or *GSTT1-1* genotype group included homozygous and heterozygous carriers of the functional allele.

iNOS genotyping

The iNOS microsatellite marker located in the promoter region, 2.5 kb upstream of the transcription start site, was amplified by PCR combined with fluorescence labeled primers as described previously (16). Amplification product sizes were estimated using capillary electrophoresis with ABI PRISM 310 automated sequencer and GeneMapper analysis software (Applied Biosystems, Norwalk, CT). Allele distribution of pentanucleotide (CCTTT)n repeats was assessed according to the different length of amplicons.

Statistical analysis

SPSS version 16.0 software (SPSS, Chicago, IL) was used. The sample size and power estimation suggested conventional statistical methods for comparison between case and control subjects and 86% power to detect median size affect (0.3–0.5) with an α level of 0.05. The null hypothesis that there is no association between the genotype and the development of diabetic retinopathy or diabetic nephropathy in type 1 diabetes was tested for each polymorphic gene locus, and *P* values <0.05 were considered statistically significant. Correction for potential multiple testing errors was performed using the Bonferroni method. Testing for deviation from Hardy-Weinberg equilibrium (HWE) using an HWE calculator was performed, and observed genotype frequencies of the studied polymorphisms were in agreement with HWE ($\chi^2 = 0.291$, $P = 0.864$ for MnSOD and $\chi^2 = 0.828$, $P = 0.363$ for GSTM1).

An independent *t* test was performed to compare the continuous parameters between case and control subjects. Lev-

Table 2—Genotype frequencies of MnSOD, GSTM1, GSTT1, and CAT polymorphisms in patients with type 1 diabetes with retinopathy or nephropathy

	Diabetic retinopathy, control 32/92 (%)	P	OR	95% CI	Diabetic nephropathy, control 37/87 (%)	P	OR	95% CI
MnSOD								
Genotype Ala/Ala	18.8/28.3	0.290	1.71	0.63–4.63	18.9/28.7	0.253	1.73	0.67–4.45
Genotype Ala/Val	46.9/54.3	0.530	0.77	0.34–1.74	62.2/48.3	0.157	1.76	0.80–3.86
Genotype Val/Val	34.4/17.4	0.045	2.49	1.00–6.16	18.9/23.0	0.615	0.78	0.30–2.05
CAT								
Genotype CC	64.5/52.7	0.255	0.61	0.26–1.43	62.2/52.9	0.346	0.68	0.31–1.51
Genotype CT	32.3/41.8	0.35	0.66	0.28–1.57	32.4/42.4	0.303	0.65	0.29–1.47
Genotype TT	3.2/5.5	0.614	1.74	0.19–15.54	5.4/4.7	0.870	0.86	0.15–4.94
GSTM1								
Genotype GSTM1-1	75.0/46.7	0.031	2.63	1.07–6.47	59.5/58.6	0.931	1.03	0.47–2.26
Genotype GSTM1-0	25.0/53.3	—	1.00		40.5/41.4	—	1.00	
GSTT1								
Genotype GSTT1-1	81.2/75.0	0.472	1.44	0.53–3.95	81.1/74.7	0.443	1.45	0.56–3.76
Genotype GSTT1-0	18.8/25.0	—	1.00		18.9/25.3	—	1.00	

ORs and 95% CIs were calculated from a cross-tabulation 2 × 2 table and risk estimate of one genotype versus the rest of all other genotypes comparing patients with type 1 diabetes with diabetic retinopathy or diabetic nephropathy and those without diabetic retinopathy or diabetic nephropathy.

ene test for equality of variances suggested which statistical analysis is more appropriate and which *P* value should be considered. Genotype and allele frequencies between study groups were compared by Pearson χ^2 and/or Fisher exact test. Strength of association between polymorphisms in genes encoding antioxidant enzymes and development of complication in diabetes was estimated using odds ratios (ORs) and 95% CI. The binary logistic regression analysis was applied to check on the calculated ORs, adjusted for independent variables such as age, sex, age at onset, and duration of diabetes.

RESULTS — Clinical characteristics of patients are presented in Table 1. No significant differences were observed between both groups regarding age, age at onset, duration of diabetes, or mean A1C.

The *MnSOD* genotype Val/Val was significantly more frequent in patients with diabetic retinopathy, carrying a 2.49-fold higher risk for development of diabetic retinopathy (OR 2.49, 95% CI 1.00–6.16, *P* = 0.045) (Table 2). Genotype *GSTM1-1* was significantly more frequent in patients with diabetic retinopathy, carrying a 2.63-fold higher risk for the development of diabetic retinopathy (OR 2.63, 95% CI 1.07–6.47, *P* = 0.031) (Table 2). Results of the Pearson χ^2 test revealed no significant differences in allele and genotype frequencies for *CAT*

and *GSTT1* polymorphisms in association with diabetic retinopathy or diabetic nephropathy.

Nine alleles for the *iNOS* microsatellite ranging from 176 to 216 bp (8–16 pentanucleotide repeats) were observed (Table 3). No significant difference in overall allelic distribution was detected between patients with diabetic retinopathy versus control subjects (χ^2 = 1.738, df = 8, *P* = 0.988) or between patients with diabetic nephropathy versus control subjects (χ^2 = 7.736, df = 8, *P* = 0.460). Allele 196 (12 repeat allele) proved to be the most frequent allele in our study population (31.2% of all observed alleles). We detected

slightly increased risk for diabetic retinopathy in patients carrying allele 196 (OR 2.19, 95% CI 0.88–5.49, *P* = 0.089), but it did not reach statistical significance.

Interaction between different genotypes in genes encoding for antioxidant enzymes was investigated (Table 4). The carriers of both *GSTM1-0* and *GSTT1-0* null genotypes, which result in a complete lack of enzyme activity, did not have an increased risk for development of microangiopathic complication in type 1 diabetes. Patients with a combination of *MnSOD* Val/Val genotype and *GSTM1-1* normal genotype had significantly higher

Table 3—Allele frequencies of iNOS (CCTTT)n gene polymorphism in patients with type 1 diabetes with diabetic retinopathy or diabetic nephropathy and control subjects

Number of (CCTTT) repeats	Size (base pairs)	Diabetic retinopathy subjects (%)	Control subjects (%)	Diabetic nephropathy subjects (%)	Control subjects (%)
8	176	1.8	1.6	0	2.4
9	181	3.6	3.3	1.4	4.2
10	186	10.7	13.6	12.2	13.3
11	191	19.6	23.4	31.1	18.7
12*	196	35.7	29.9	29.7	31.9
13	201	16.1	17.4	16.2	17.5
14	206	8.9	8.2	8.1	8.4
15	211	3.6	2.2	1.4	3.0
16	216	0	0.5	0	0.6

Four patients were excluded from the analysis because of insufficient PCR amplification of the iNOS microsatellite locus. *P* values are the result of Pearson χ^2 test comparing allele versus all the other alleles. *OR 2.19, 95% CI 0.88–5.49, *P* = 0.089.

Table 4—*Genotype frequencies for gene–gene interactions between patients with type 1 diabetes with retinopathy or without retinopathy (control subjects)*

	Diabetic retinopathy subjects, $n = 32$ (%)	Control subjects, $n = 92$ (%)	P	OR	95% CI
GSTM1–1 and *GSTT1–1*					
Carriers	53.1	35.9	0.087	2.03	0.89–4.58
Noncarriers	46.9	64.1			
MnSOD Val/Val and *GSTM1–1*					
Carriers	41.7	14.3	0.009	4.24	1.37–13.40
Noncarriers	58.3	85.7			
MnSOD Val/Val and *GSTT1–1*					
Carries	30.8	17.4	0.154	2.11	0.75–5.97
Noncarriers	69.2	82.6			

ORs and 95% CIs were calculated from cross-tabulation 2 × 2 table composing carrier versus noncarrier of particular genotype among patients with type 1 diabetes with retinopathy and without retinopathy.

risk for retinopathy than patients lacking this particular combination (OR 4.24, 95% CI 1.37–13.40, $P = 0.009$).

CONCLUSIONS — We hypothesized that genetic variability of enzymes regulating oxidative stress could be involved in development of microangiopathic complications in people with type 1 diabetes.

The *MnSOD* Val/Val genotype was associated with a 2.49-fold higher risk for diabetic retinopathy in our cohort of patients with type 1 diabetes. Val/Val genotype combined with smoking is associated with diabetic nephropathy in type 1 diabetes (7). We did not find an association between Val/Val and diabetic nephropathy in our cohort of patients with type 1 diabetes where none reported smoking. *MnSOD* Val16Ala polymorphism is also associated with diabetic retinopathy (8) and diabetic nephropathy (17) in type 2 diabetes. In vitro studies show that amino acid substitution of Ala with Val modifies helical structure of the signal sequence and alters the import of *MnSOD* enzyme into the mitochondrial matrix (18). Less efficient import decreases *MnSOD* concentration inside mitochondria and results in overproduction of superoxide, which is considered to be a causal link between hyperglycemia and metabolic pathways involved in vascular complications in diabetes (4). Studies on mice, treated with streptozotocin, show that MnSOD overexpression in mitochondria play a significant protective role in development of diabetic retinopathy (19).

A significant association between the presence of the functional *GSTM1* gene and development of diabetic retinopathy was observed in our study. This suggested that deletion in the *GSTM1* gene had a protective role for diabetic retinopathy. A large number of studies on *GSTM1-0* and/or *GSTT1-0* null genotypes report an increased risk for development and progression of rheumatoid arthritis and asthma (20,21). However, one study reports an association of *GSTM1* gene deletion with protection from development of type 1 diabetes in a group of 14- to 20-year-old children (11). The protective role of *GSTM1* null genotype in the development of diabetic retinopathy in type 1 diabetes has, to the best of our knowledge, not been described yet. One possible explanation for the protective role is that the absence of the *GSTM1* gene may upregulate other antioxidant enzymes including MnSOD (22). However, all explanations are speculative and need to be confirmed in further studies.

When studying the synergistic effect of different polymorphisms, we observed a 4.24-fold higher risk for development of diabetic retinopathy in patients with type 1 diabetes carrying the *MnSOD* Val/Val and normal *GSTM1-1* genotype. Evidence of interaction between MnSOD and GST genes is reported in a case-control study of rheumatoid arthritis (23) but to our knowledge has not been described in patients with type 1 diabetes and diabetic retinopathy.

The association studies that evaluate the impact of genotype on disease progression are usually limited by the fact that more chronic complications will develop with longer follow-up. Considering that the duration of diabetes is an important risk factor for microangiopathic com-

plications, our results must be interpreted with caution. In most patients with type 1 diabetes, the earliest signs of diabetic complications occur after 5–10 years, and the highest incidence (between 75 and 95%) occur after 10 years (24). Control subjects in our study were without diabetic complications for a mean of 17.9 years after the onset of type 1 diabetes. Another limitation of our study was a relatively small number of participants. However, the studied population was homogenous, and power estimation showed that our study had an 86% power to detect median size affect (0.3–0.5) with an α level of 0.05.

Other genetic polymorphisms involved in the hyperglycemia-induced cell damage could influence our results. Notably, advanced glycation end products modify ROS formation through advanced glycation end product receptors and therefore influence the production of growth factors and cytokines by affected cells (4). Our results thus represent only a part of the complex pathobiologic network of diabetic retinopathy or diabetic nephropathy.

Finally, poor glycemic control increases the risk for diabetic nephropathy or diabetic retinopathy. Moreover, a limited period of poor glycemic control can have a prolonged effect on the incidence of diabetic retinopathy ("metabolic memory") as demonstrated by the Epidemiology of Diabetes Interventions and Complications (EDIC) cohort follow-up (25). Our groups with and without diabetic retinopathy had similar mean A1C. However, possible periods of poor glycemic control in individual patients during the observation period could influence our results.

In conclusion, we found a statistically significant association between *MnSOD* Val/Val and *GSTM1-1* genotype and development of diabetic retinopathy in type 1 diabetes. To the best of our knowledge, this is the first study reporting a positive association of a normal *GSTM1-1* genotype with development of a microangiopathic complication in type 1 diabetes. Additionally, a combination of both *MnSOD* Val/Val and *GSTM1-1* genotypes further increased the risk for diabetic retinopathy. Testing for genetic markers in genes encoding antioxidant enzymes either individually or in combination could be added to a genetic panel for identifying patients at higher risk for developing diabetic retinopathy in clinical practice.

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