Association investigation of *BACH2* rs3757247 and *SOD2* rs4880 polymorphisms with the type 1 diabetes and diabetes long-term complications risk in the Polish population

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Abstract. Genetic factors are indicated in the development of type 1 diabetes (DM1). Recently, nucleotide variants of *BACH2* and *SOD2* have been associated with this chronic condition. Therefore, the purpose of the present study was to investigate the contribution of *BACH2* rs3757247 and *SOD2* rs4880 (Ala16Val) polymorphisms to the risk of DM1 and diabetes long-term complications. Selected polymorphic variants of *BACH2* and *SOD2* were investigated in a group of 141 patients with DM1 and in a group of age, gender-matched healthy subjects (n=369) using a high-resolution melting curve method. There was no evidence for either allelic or genotypic association with the risk of DM1 and diabetes chronic complications for analysed polymorphisms. In addition, no interaction between *BACH2* and *SOD2* variants in the development of this condition was observed. However, the frequency of *BACH2* rs3757247 AG and AA genotypes was statistically different between DM1 patients with retinopathy and healthy individuals (odds ratio, 2.455; 95% confidence interval, 0.999-6.035; P=0.044), but this result did not survive multiple testing corrections. The present study did not confirm the involvement of *BACH2* rs3757247 and *SOD2* rs4880 polymorphisms in the development of DM1 and diabetes long-term complications. Further studies in a larger population sample are required.

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Introduction

Type 1 diabetes (DM1) is a multifactorial disease that results from autoimmune destruction of β-cells in the pancreas (1). DM1 characterises the largest range of concordance rates in monozygotic twins among all autoimmune diseases, and this provides convincing evidence that genetic factors are strongly involved in the pathogenesis of DM1 (2). Initially, based on family-based linkage analyses, the strong association between human leukocyte antigen (HLA) class II genes encoding cell-surface antigen-presenting proteins and DM1 was found (3). With the advent of high-throughput single-nucleotide polymorphism (SNP) genotyping array technology studies, searching for the novel DM1 loci became possible (4). Based on the results of these studies, >60 new loci were identified across the human genome, which were associated with the risk of DM1 (5). In addition, advanced genetic technologies have become an important strategy to identify genetic factors contributing to the development of chronic diabetic complications (6,7).

Recently, follow-up analysis of a genome-wide association study was performed on DM1 patients and revealed that an SNP in the intron of the BTB and CNC homology 1, basic leucine zipper transcription factor 1 (*BACH2*) gene (rs3757247) was associated with the risk of DM1 (8).

The protein product of *BACH2* plays a role as a key regulator of nucleic acid-triggered antiviral responses in human cells (9). It has been shown that Bach2 is expressed in the early stages of B-cell differentiation and is suppressed in terminally differentiated plasma cells by repressing the expression of B lymphocyte-induced maturation protein-1, the key regulator

of plasma cell differentiation. The observed expression profile may serve to sensitize cells to oxidative stress and/or allow for the effective elimination of cells at early stages of differentiation (10). *BACH2* is also critical for class switch recombination and somatic hyper mutations (SHM) of immunoglobulin genes. Analysis of transgenic Bach2$^{-/-}$ mice revealed that *BACH2* is a key regulator of antibody response, which is required for T-cell independent and dependent immunoglobulin G (IgG) responses and SHM (11). Additionally, *BACH2* may contribute to the development of DM1 by regulation of proinflammatory cytokine-induced apoptotic pathways in pancreatic β-cells by crosstalk with the *PTPN2* gene and activation of *JNK1* and *BIM* (12).

The missense T to C nucleotide gene variant of *SOD2* (rs4880) is another SNP associated with the risk of DM1 and its chronic complications (13). The gene product of *SOD2* is the free radical scavenging mitochondrial SOD2 that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide (14). The presence of the C allele at the -9 position of the *SOD2* gene results in a substitution of alanine for valine (Ala16Val) in the mitochondrial targeting sequence of the enzyme. This substitution is associated with less efficient transport of SOD2 into the mitochondrial matrix and decreases the efficiency of SOD2 to neutralise superoxide radical (15). The production of superoxide in DM1 is enhanced by frequent episodes of hyperglycaemia (16). This results in the dysfunction and apoptosis of endothelial cells, which contribute to the development of chronic complications (17). Therefore, the *SOD2* CC variant may increase the risk of DM1 and its chronic complications, which was shown in certain clinical studies (18,19).

Based on the results of the studies showing the significance of the association between the *BACH2* gene rs3757247 and *SOD2* gene rs4880 polymorphisms and risk of DM1 and diabetes chronic complication pathogenesis (8,15), the analysis of the distribution of selected polymorphic variants of *BACH2* and *SOD2* in a Polish DM1 population was performed.

Materials and methods

Study group. The study group was composed of 141 DM1 patients from the Department of Internal Medicine and Diabetology, Poznan University of Medical Sciences (Poznan, Poland). DM1 in all the patients was diagnosed according to the criteria from the American Diabetes Association on the basis of classical symptoms, blood glucose concentration >11.1 mmol/l and C-peptide concentration <0.5 μg/l (20). The mean age of DM1 patients was 33±10 years and the mean diabetes duration was 15±8 years. All the patients underwent a screening for diagnosis of microvascular complications, such as diabetic retinopathy, diabetic kidney disease and diabetic neuropathy.

Diabetic retinopathy was diagnosed by direct ophthalmoscopy through dilated pupils followed by fundus photography in all the patients. Diabetic retinopathy was graded according to the classification of the American Academy of Ophthalmology into classifications of: No retinopathy; mild, nonproliferative retinopathy (mild, moderate and severe); and proliferative retinopathy (21).

Diabetic kidney disease was detected at the stage of albuminuria (urinary albumin excretion rate between 30 and 300 mg/24 h) in two samples collected over a 3-month period after exclusion of secondary causes of microproteinuria. Diabetic kidney disease was defined as the presence of albuminuria in connection with diabetes of >10 years in duration or with diagnosed diabetic retinopathy (22).

Diabetic neuropathy was diagnosed in patients with two or more of the following components: The presence of neuropathy symptoms, the absence of ankle tendons on reflexes and abnormal scores for pressure and/or vibration perception (20).

The control group consisted of 369 healthy, age- and gender-matched individuals randomly selected from blood donors and healthy volunteers. All the study participants were Caucasians of Polish origin. The study received approval from the Ethics Committee of Poznan University of Medical Sciences (no. 607/12) and written informed consent was obtained from all the participants.

Biochemical parameters. Analysis of biochemical parameters was performed in an accredited medical laboratory. The serum concentration of total cholesterol (TCH) was determined using the commercially available assay kits (Roche Diagnostics GmbH, Mannheim, Germany). The glycated hemoglobin A1c (HbA1c) value was measured using high-performance liquid chromatography with the Variant Hemoglobin A1c Program (Bio-Rad, Hercules, CA, USA) (23).

SNP selection and genotyping. *BACH2* rs3757247 and *SOD2* rs4880 polymorphic variants were selected based on their association with DM1 and diabetes complications in previous studies. Characteristics of those SNPs are presented in Table I. DNA was isolated from peripheral blood lymphocytes by salt extraction. Genotyping was carried out by high-resolution melting (HRM) curve analysis on the LightCycler® 480 system (Roche Diagnostics GmbH) with the use of the 5x HOT FIREPol® EvaGreen® HRM mix (Solis BioDyne, Tartu, Estonia). For quality control, ~10% of the randomly chosen samples were re-genotyped. Samples that failed genotyping were not repeated and were excluded from statistical analyses. Primer sequences and conditions for HRM analysis are presented in Table II.

Statistical analysis. For each SNP, the Hardy-Weinberg equilibrium was assessed by the χ^2 test in patients and controls and $P<0.05$ was considered to indicate a statistically significant difference. The differences in allele and genotype frequencies between cases and controls were determined using standard χ^2 or Fisher tests. SNPs were tested for the association with DM1 or chronic diabetic complications using the Cochran-Armitage trend test. The strength of the association was measured by odds ratios (ORs) with 95% confidence intervals (CIs). The data were analysed under recessive and dominant inheritance models. A statistical adjustment for multiple comparisons was accomplished using the Bonferroni correction.

Statistical significance was interpreted as $P<0.05$. The gene-gene interaction among tested SNPs was analysed using the logistic regression and epistasis option in the PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/). PLINK

Table I. Characteristics of the polymorphisms genotyped in the *SOD2* and *BACH2* genes.

Gene symbol	Chromosome location	rs number	SNP function	Alleles[a]	MAF[b]
BACH2	6q15	rs3757247	intron	A/G (REV)	0.47
SOD2	6q25.3	rs4880	missense (p.Val16Ala)	C/T (REV)	0.46

[a]According to the Single Nucleotide Polymorphism database (dbSNP); underlining denotes the minor allele. [b]MAF from 1,000 genomes project for EUR samples. *BACH2*, basic leucine zipper transcription factor 2; *SOD2*, superoxide dismutase 2; SNP, single-nucleotide polymorphism.

Table II. High resolution melt analysis conditions for the identification of the polymorphisms genotyped in the data set.

Gene symbol	rs number	Primers for PCR amplification (5'-3')	Annealing temp., ˚C	PCR product length, bp	Melting temp. range, ˚C
BACH2	rs3757247	F: 5'TCACCATCATCCACCCTATG3' R: 5'TTCTAGGCATGGGAACCACT3'	57.7	103	76-84
SOD2	rs4880	F: 5'GCTGTGCTTTCTCGTCTTCAG3' R: 5'CCGTAGTCGTAGGGCAGGT3'	60.6	105	82-92

PCR, polymerase chain reaction; bp, base pair.

generates a model based on allele dosage for each SNP and considers allelic by allelic epistasis. Values of P<0.05 were considered to indicate a statistically significant difference. To assess which parameter independently influences the presence of chronic complications in DM1 patients, multivariable regression analysis was performed as follows: Age of DM1 verses HbA1c level verses DM1 duration verses variants of *BACH2* rs3757247 and *SOD2* rs4880 polymorphisms.

Results

Patient characteristics. The basic characteristics of the patients are presented in Table III. There were 81 females and 60 males among the Polish patients with DM1. The mean of TCH concentration was within the reference level [4.0 (3.9-4.4) mmol/l], whereas the mean level of HbA1c [8.0 (7.1-8.9) %] showed that the majority of the patients did not maintain good glycaemic control.

Retinopathy was diagnosed in 38 patients (27%), diabetic kidney disease in 8 patients (6%) and neuropathy in 14 patients (10%). Of these patients, 25 had retinopathy only (18%), 4 patients had only neuropathy (3%) and 16 patients (11%) had more than one microvascular complication.

Associations of the tested polymorphisms. None of the tested polymorphisms showed evidence of deviation from Hardy-Weinberg equilibrium in either DM1 patients or controls (P>0.05). Genotype counts, genotypic OR and corresponding 95% CI calculations for *BACH2* rs3757247 and *SOD2* rs4880 are reported in Table IV. There was no evidence for either allelic or genotypic association with the risk of DM1 for the analysed nucleotide variants. The minor allele frequency was 0.48 in cases and 0.44 in controls for *BACH2* rs3757247 (pallelic=0.327), and 0.48 (allele T) in cases and 0.48 (allele C) in controls for *SOD2* rs4880 (pallelic=0.332). The calculated

Table III. Basic characteristics of the DM1 patients.

Characteristics	DM1, n=141	Control, n=369
Gender, F/M	81/60	200/169
Age, years	33±10	35±10
DM1 duration, years	15±8	
BMI, kg/m^2	24±3	
TCH, mmol/l	4.0 (3.9-4.4)	
HbA1c, %	8.0 (7.1-8.9)	

DM1, type 1 diabetes; F, female; M, male; BMI, body mass index; TCH, total cholesterol level; HbA1c, glycated hemoglobin A1c.

OR for individuals with the combined AA and AG genotypes of the *BACH2* rs3757247 polymorphism compared to the GG homozygotes was 1.554 (95% CI, 0.989-2.441). However, this result was not statistically significant (P=0.055, pcorr=0.110).

The gene-gene interaction analysis showed no significant epistatic interaction between the *BACH2* and *SOD2* variants in the development of DM1 (Table V). The OR for interaction was 1.066 (P=0.763).

No significant association between the *BACH2* rs3757247 or *SOD2* rs4880 genotypes with different chronic diabetic complications was identified (Table VI). However, there was an exception of the borderline association with diabetic retinopathy for the *BACH2* rs3757247 variant. Compared to the GG homozygotes, individuals with the AG or AA genotypes (dominant model of inheritance) had an OR of 2.455 (95% CI, 0.999-6.035; P=0.044). However, the significance of this result did not survive multiple testing corrections

Table IV. Association of polymorphic variants of the *SOD2* and *BACH2* genes with the risk of diabetes.

Gene	rs number	Alleles[a]	Genotypes cases[b]	Genotypes controls[b]	P_{trend} value	$P_{genotypic}$ value	$P_{allelic}$ value	$OR_{dominant}$ (95% CI)[c]; P-value	$OR_{recessive}$ (95% CI)[d]; P-value
BACH2	rs3757247	A/G	32/82/26	116/177/75	0.320	0.084	0.327	1.554 (0.989-2.441); 0.055	0.891 (0.543-1.463); 0.648
SOD2	rs4880	C/T	32/72/37	98/185/86	0.330	0.613	0.332	1.232 (0.780-1.945); 0.371	1.171 (0.749-1.829); 0.488

[a]Underline denotes the minor allele in the control samples; [b]genotype order: DD/Dd/dd (d is the minor allele in the control samples); [c]dominant model: dd+Dd vs. DD (d is the minor allele); [d]recessive model: dd vs Dd+DD (d is the minor allele) CI, confidence interval; OR, odds ratio.

Table V. Results of the gene-gene interaction analysis.

Gene 1	SNP 1	Gene 2	SNP 2	OR for interaction	χ^2 statistic	1df asymptotic P-value
BACH2	rs3757247	*SOD2*	rs4880	1.066	0.091	0.763

SNP, single-nucleotide polymorphism; OR, odds ratio; df, degrees of freedom.

Table VI. Association of the polymorphic variants of *SOD2* and *BACH2* with the risk of diabetic complications.

SOD2 rs4880	TT	CT	CC	OR (95% CI); P-value CC+CT vs. TT	OR (95% CI); P-value CC vs. CT+TT
Controls	98	185	86		
Patients					
Diabetic complications	11	21	13	1.118 (0.545-2.292); 0.761	1.337 (0.672-2.661); 0.407
Retinopathy	10	17	11	1.013 (0.474-2.161); 0.974	1.341 (0.639-2.815); 0.437
Diabetic kidney disease	4	3	1	0.362 (0.089-1.474); 0.220[a]	0.470 (0.057-3.876); 0.688[a]
Neuropathy	5	8	3	0.796 (0.270-2.348); 0.678	0.759 (0.211-2.728); 1.000[a]
BACH2 rs3757247	GG	AG	AA	OR (95% CI); P-value AA+AG vs. GG	OR (95% CI); P-value AA vs AG+GG
Controls	116	177	75		
Patients					
Diabetic complications	9	25	11	1.841 (0.859-3.949); 0.112	1.264 (0.612-2.612); 0.526
Retinopathy	6	24	8	2.455 (0.999-6.035); 0.044	1.042 (0.459-2.366); 0.922
Diabetic kidney disease	3	3	2	0.767 (0.180-3.266); 0.712[a]	1.302 (0.258-6.585); 0.669[a]
Neuropathy	4	7	5	1.381 (0.436-4.375); 0.784[a]	1.776 (0.599-5.268); 0.295

Comparisons of *SOD2* and *BACH2* genotypes between patients with a particular diabetic complication and controls was performed by χ^2 test. [a]Fisher exact test. OR, odds ratio; CI, confidence interval.

(pcorr=0.088). The result of multivariate regression analysis has shown that only the duration of DM1 will independently influence the presence of chronic complications in DM1 patients (β=0.473, R^2=0.300, P<0.05).

Discussion

Previous results of large meta-analysis studies show that the *BACH2* rs3757247 and *SOD2* rs4800 polymorphisms played

a role in the development of DM1 (8,13). The A allele of rs3757247 polymorphism in *BACH2* increased the risk of DM1 (8), whereas the C allele of the rs4880 polymorphism in the *SOD2* gene had protective effects on the risk of DMI (13). In the present study, this was inconclusive. Most likely, one of the reasons of a negative association between the rs37577247 polymorphism in *BACH2* and DM1 risk is the extremely modest contribution of non-HLA loci to the development of DM1 associated with the HLA region (24). In addition, it was shown that the rs3757247 polymorphism of *BACH2* facilitated autoimmunity rather than increased the risk of DM1 itself (25,26). In the genome-wide association analysis of autoantibody positivity in DM1 patients, the association between the minor allele of the *BACH2* rs3757247 variant and higher positivity of thyroid peroxidase autoantibodies was identified (25). This led to the conclusion that this polymorphic variant increased the risk of Graves' disease (26), not DM1 itself. Additionally, it was shown that $Bach2^{-/-}$ mice present deficient T-cell-independent and dependent IgG responses, which shows that Bach2 is important for the immune reaction and played an important role in autoimmune response (11).

Notably, the rs3757247 polymorphism of *BACH2* may be associated with a risk of retinopathy. This finding may arise from the role of *BACH2* in the induction of apoptosis under oxidative stress. *BACH2* is a proapoptotic factor as it inhibits anti-oxidative and anti-apoptotic genes in the response of enhanced production of reactive oxygen species (ROS) (27). Oxidative stress is associated with the diabetic condition and is one of the most common mechanisms leading to the development of retinopathy (28), therefore we believe that the rs3757247A allele of *BACH2* could be involved in enhanced apoptosis of retinal cells. However, the significance of the present result did not survive multiple testing corrections. Consequently, an additional large-scale study should be performed to conclude whether the rs3757247 polymorphism of *BACH2* represents novel loci in the pathogenesis of DM1 and/or diabetes retinopathy.

Thus far, there is only one study showing the association of the rs4880 polymorphism of *SOD2* with the risk of DM1 (12). The majority of the studies indicate that this polymorphism impacted the development of microvascular complications in patients with diabetes (29-31). The Val/Val genotype of rs4880 was shown to increase the risk of retinopathy in DM1 patients; however, no association was found for nephropathy (29). Notably, studies performed on Finish and Swedish populations of DM1 patients indicated that the homozygosity for the *SOD2* Val allele is associated with an increased risk of nephropathy (30). Similar results were shown for Japanese type 2 diabetic patients (31). In the present study, no associations of the *SOD2* rs4880 polymorphism with any of the diagnosed microvascular complications in the studied group were identified. These observations are consistent with the findings of el-Masry *et al* (19), who demonstrated that the Val/Val genotype of rs4880 is not a significant factor in diabetic patients with nephropathy. In addition, no association was identified between rs4880 polymorphisms and the risk of microvascular complications in DM1 or DM2 patients. Notably, the association between macroangiopathy and the lower frequency of the C (Ala) allele of *SOD2* rs4880 was identified in this study (32). The Val isoform of *SOD2* may lead to a decreased resistance against ROS produced in the mitochondria and to oxidative damage of proteins caused by less efficient SOD2 transport into the mitochondria (33). Therefore, we expected to find the association between the TT genotype of the *SOD2* gene and the presence of long-term complications, caused partially by enhanced oxidative stress (28). One of the explanations for the negative result could be due to a relatively small sample size of the DM1 group. It has to be considered that due to this limitation, the statistical analysis may have failed to demonstrate any significant differences. Another reason of failure to replicate an association of SNPs for candidate genes could be too low a number of selected SNPs to achieve full gene coverage.

By contrast, the result of linear regression showed that the DM1 duration independently influenced the development of microvascular complications in DM1 patients, indicating the conventional risk factors as: Diabetes duration and high glycosylated level play a primarily role in the development of microangiopathy in DM1 (34).

In conclusion, neither *BACH2* rs3757247 nor *SOD2* rs4880 increase the risk of DM1 or chronic diabetic complications in Polish DM1 patients. To confirm this result, further investigations on larger groups should be performed.

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