

No Association Between Antioxidant Enzyme Gene Polymorphism and Albuminuria in Type 2 Diabetes Mellitus Cases

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Diabetic nephropathy is a frequent microvascular complication of type 2 diabetes mellitus (T2DM) and it represents one of the main causes of end stage renal disease its initial manifestation consisting in increased urinary excretion of albumin (albuminuria) which can slowly progress to overt albuminuria and renal failure. Susceptibility to develop diabetic nephropathy is modulated by genetic factors and oxidative stress play an important role. We investigated the relation between polymorphisms in genes related to oxidative stress and the presence of albuminuria in T2DM patients. Blood samples for biochemical and genetic analysis were collected from 98 patients with T2DM. Albuminuria was analyzed from a 12 h overnight urine sample and was graded after the value of albumin to creatinine ratio (ACR) as normoalbuminuria (0-30 mg/g) and albuminuria (ACR > 30 mg/g) and patients with persistent albuminuria were included in the group with albuminuria. In our study we found a statistically significant association between peripheral neuropathy and the presence of albuminuria ($p=0.005$) and statistically significant association between low HDL-cholesterol level and the presence of albuminuria ($p=0.02$). GSTM1, GSTT1, GSP1 Ile105Val, GPX1 Pro198Leu, CAT C262T polymorphism was not associated with the presence of albuminuria in T2DM patients. The present study suggests that GSTM1, GSTT1 and GSP1 Ile105Val, GPX1 Pro198Leu and CAT C262T gene polymorphisms are not associated with the susceptibility of the diabetic patients to develop albuminuria.

Key words: oxidative stress, albuminuria, diabetes mellitus, CAT C262T, GPX1

Diabetic nephropathy (DN) is an important microvascular complication of diabetes mellitus and it represents one of the main causes of end stage renal disease and an early cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM). Its initial manifestation consists of increased urinary excretion of albumin (albuminuria) which can slowly progress to overt albuminuria and renal failure [1].

Oxidative stress plays an important role in the pathogenesis of several diseases (tumors, ophthalmological disorders, leukemia, coronary artery disease, diabetes mellitus) and leads to alterations of the mitochondrial DNA [2 - 5]. T2DM is associated with a high oxidative stress and free radical-induced lipid peroxidation which can play a significant part in the genesis of microvascular complications, including DN. A decrease in the antioxidant capacity destabilizes the equilibrium between the oxidation and antioxidation. In vivo studies described a series of enzymes that hold an antioxidant capacity, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), nitric oxide synthase (NOS), enzymes that play a key role in preventing the lesions induced by ROS [6].

Even though it is clear that a poor glycemic control is the major cause of microvascular complications in patients with DN, susceptibility to develop DN is modulated by genetic factors. [7, 8].

Catalase is an antioxidant enzyme which represents the first line of defense against the oxidative stress [9] with an important role in redox regulation in the kidneys

when hyperglycemia is present. It also mediates the reduction of oxygen peroxide (H_2O_2) to water and oxygen and represents an important part in developing tolerance for the oxidative stress [7, 10, 11]. Catalase is an ubiquitous enzyme which is found in all the aerobic organisms and is present in high levels in the liver, kidneys and erythrocytes [9]. One of the CAT gene polymorphisms, known as C262T has been associated with a reduction of the catalytic activity which leads to an increase in ROS levels [12].

Glutathione peroxidase (GPX) is an antioxidant enzyme with a high expression in the blood vessels, with a primordial role in protecting the cells against the oxidative stress by reducing the hydrogen peroxide (H_2O_2) and other organic peroxides to water with reduced glutathione [13]. One of the GPX enzymes is GPX1, a selenium dependent enzyme, its gene being located on 3p21 chromosome. One of its polymorphisms consists of leucine to proline substitution at codon 198 [14]. This amino acid substitution leads to a decrease in the enzymatic activity of GPX.

Glutathione-S-transferases (GSTs) represent a family of multifunctional isoenzymes with antioxidant function with an important function against ROS (reactive oxygen species) and are implicated in cell protection and detoxification [1]. Genetic polymorphisms can reduce their antioxidant activity with increased oxidative stress which is implicated in the etiopathogenesis of microvascular chronic diabetes complications. GSTM1 null and GSTT1 null genotypes determine the enzymatic inactivity of the

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Table 1
BASELINE CHARACTERISTICS OF STUDIED PATIENTS

Variable (ACR) mg/g	0-30	>30	p value
Patients number (%)	74 (75.5%)	24 (24.5%)	
Female, No (%)	44 (59.5%)	14 (58.3%)	0.92
Age (years)	62.6±8.8	66.3±10.3	0.08
Height (cm)	167±7.1	167.7±8.2	0.67
Weight (kg)	84.7±13.9	83.7±15.5	0.77
Age at diagnosis (years)	54.3±10.1	56.3±9.4	0.41
Diabetes duration (years)* median (min-max)	7 (1-29)	9 (1-20)	0.29
Neuropathy, No (%)	31/41.9%	18/75%	0.005*
Body mass index (kg/m ²)	30.4±4.6	30±5.4	0.60
Hypertension (yes), No (%)	71/95.9%	22/91.7%	0.40
Systolic blood pressure (mmHg)	150.6±22.6	160.6±33.7	0.10
Diastolic blood pressure (mmHg)	88.9±11.3	92±21.3	0.36
Creatinine (mg/dL)	0.89±0.24	0.99±0.33	0.10
Blood urea nitrogen (mg/dL)	37.8±12.9	46.3±18.2	0.01*
HDL cholesterol (mg/dL)	43.3±11.8	37.1±10.9	0.02*
LDL cholesterol (mg/dL)	106.2±38.2	109.6±62.7	0.75
Total cholesterol (mg/dL)	181.9±48.4	180.3±72.6	0.90
Triglycerides (mg/dL)	161.9±98.0	168.2±77.3	0.75
HbA1c (%)	7.20±1.27	7.48±2.00	0.42
Statins (yes), No (%)	45/60.8%	13/54.2%	0.56
Fibrates (yes), No (%)	3/4.1%	0/0.0%	0.31
Oral hypoglycemic agents (yes), No (%)	59/79.7%	20/83.3%	0.69
Insulin (yes), No (%)	30/40.5%	12/50.0%	0.41
eGFR (estimated glomerular filtration rate)	82.5±19.6	76.5±30.4	0.25

Data were expressed as: mean±SD – Student test; median (min-max) – Mann Whitney test; No (%) - Chi square test, Fisher's test. *Shows significant statistical difference between lots

proteins encoded, leading to the association of these variants with diseases such as DM and its complications [15].

The purpose of this research was to establish the frequency of five polymorphisms in GST genes (namely GSTM1, GSTT1 and GSTP1 Ile105Val), GPX1 (namely Pro198Leu) and CAT (namely C262T) genes in T2DM patients with/without albuminuria and to evaluate the association of these polymorphisms with albuminuria in T2DM patients.

Experimental part

Material and method

Study population

All the participants included in this study were given and signed an informed consent for blood collection, biochemical and genetic analysis and the study protocol was approved by the Ethic Committees of the Emergency County Hospital from Tirgu Mures and University of Medicine and Pharmacy from Tirgu Mures, according to the principles of the Helsinki Declaration.

The patient group consisted of 98 consecutive unrelated cases, over 18 years old, diagnosed with T2DM (59.18% women and 40.82% men), which were recruited at Mures Emergency County Hospital, from August 2014 until the end of July 2015. The exclusion criteria were: urinary tract infections, hematuria, congestive heart failure, intense physical training, pregnant and lactating female and other renal pathology. The following clinical data were collected: weight, height, disease duration, systolic and diastolic blood pressure and type of hypoglycemic treatment. Albuminuria was analyzed from a 12 h overnight urine sample and was graded after the value of albumin to creatinine ratio (ACR) as normoalbuminuria (0-30 mg/g) and albuminuria (ACR > 30 mg/g). Measurements of

nighttime urine were performed and only T2DM patients with persistent albuminuria were selected in the group with albuminuria. After a prior overnight fasting, we collected venous blood samples for biochemical and genetic analysis. We used the direct method to determine LDL-cholesterol when triglyceride levels were above 400 mg/dL.

Genotyping of GST T1, GST M1, GSTP1 Ile105Val, GPX1 Pro198Leu and CAT C262T gene polymorphism

Genomic DNA was rapidly isolated from whole blood samples collected on EDTA vacutainers by using the Quick-gDNA MiniPrep kit based on Zymo-Spin technology (ZymoResearch). A multiplex polymerase chain reaction described by Sharma et al. [16] was performed for genotyping the GSTM1 and GSTT1 gene polymorphisms. In the case of GSTP1 Ile105Val, CAT C262T, GPX1 Pro198Leu gene polymorphisms, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method was performed using specific primers, PCR protocol and FastDigest enzymes (from Thermo Scientific) as previously described [17-19].

Statistical analysis

GraphPad InStat 3 software was used for statistical analyses, and a significance threshold alpha of 0.05 was used. Data were considered as nominal or quantitative variables. Nominal variables were characterized using frequencies. Quantitative variables were tested for normality of distribution using the Kolmogorov-Smirnov test and were characterized by median and min-max or by mean and standard deviation (SD), when appropriate. A chi-square or a Fisher's test were used in order to compare the frequency of normal variables. Quantitative variables were compared using the t-test or Mann-Whitney test,

Table 2

GENOTYPE DISTRIBUTION FOR THE FIVE POLYMORPHISMS INVESTIGATED IN PATIENTS WITH T2DM WITH AND WITHOUT ALBUMINURIA

Gene polymorphism	>30 n -24 (%)	<30 n - 74 (%)	p value, OR (95% CI) >30 versus <30
GPX1 Pro198Leu			
Pro/Pro	2 (8.3)	14 (18.9)	Reference
Pro/Leu	14 (58.3)	26 (35.1)	0.11, 3.76 (0.74-19.01)
Leu/Leu	8 (33.3)	34 (45.9)	0.71, 1.64 (0.31-8.75)
Pro/Leu + Leu/Leu	22 (91.6)	60 (81.1)	0.34, 2.56 (0.54-12.2)
CATC262T			
CC	10 (41.7)	35 (47.3)	Reference
CT	9 (37.5)	25 (33.8)	0.19, 1.26 (0.44-3.55)
TT	5 (20.8)	14 (33.8)	0.12, 1.25 (0.336-4.31)
CT + TT	14 (58.3)	39 (52.7)	0.23, 1.25 (0.49-3.18)
GST P1Ile105Val			
Ile/Ile	14 (58.4)	46 (62.2)	Reference
Ile/Val	5 (20.8)	21 (28.4)	0.17, 0.78 (0.25-2.45)
Val/Val	5 (20.8)	7 (9.4)	0.18, 2.34 (0.64-8.56)
Ile/Val + Val/Val	10 (41.6)	28 (37.8)	0.73, 1.17 (0.45-2.99)
GST T1			
Present	19 (79.2)	64 (86.5)	Reference
Null	5 (20.8)	10 (13.5)	0.38, 1.68 (0.51-5.53)
GST M1			
Present	10 (41.7)	36 (48.6)	Reference
Null	14 (58.3)	38 (51.4)	0.55, 1.32 (0.52-3.36)

when appropriate. Deviations of allelic frequencies from Hardy-Weinberg equilibrium were calculated using a chi-square test. Odds ratio was calculated at a 95% confidence interval. Multivariate analysis was carried out using linear regressions. We used as dependent variable the presence of albuminuria, and as independent variables polymorphisms (GSTM1, GSTT1, GSTP1 Ile105Val, GPX1 Pro198Leu, CAT C262T).

Results and discussions

Based on albumin to creatinine ratio we divided our cases in two groups: those with normoalbuminuria (0-30 mg/g) and those with persistent albuminuria (ACR > 30 mg/g).

The main characteristics of patients T2DM included in the study are summarized in table 1.

The frequency of albuminuria (ACR>30mg/g) was 24.5%. Diabetic peripheral neuropathy was more common in patients with albuminuria compared with the patients without albuminuria (75 and 41.9%, respectively) (p=0.005). Blood urea nitrogen level in patients with albuminuria was statistically significant greater compared to the ones with normal albuminuria (p=0.01). The average of HDL cholesterol level in patients without albuminuria was statistically higher compared with the ones with albuminuria (p=0.02).

Genotype distribution for the GSTM1, GSTT1, GSTP1 Ile105Val, GPX1 Pro198Leu, CAT C262T gene polymorphisms investigated in patients with T2DM with and without albuminuria are summarized in table 2.

We found no significant difference in terms of GSTM1, GSTT1, GSTP1 Ile105Val, GPX1 Pro198Leu and CAT C262T genotypes between diabetic patients with and without albuminuria (P>0.05 for all comparison performed). The combination of two variant genotypes between GSTM1, GSTT1, GSTP1 Ile105Val, GPX1 Pro198Leu and CAT C262T showed no increased risk in developing albuminuria in diabetic patients.

In addition, we examined the association between the triple combination of the mentioned gene polymorphisms and albuminuria but we found that the presence of three variant genotype in investigated gene polymorphisms in T2DM cases had no effect on the appearance of albuminuria. We performed also a binary logistic regression

where the dependent variable was the presence of albuminuria and the independent variables were GSTM1 (OR: 1.33, 95%CI: 0.50-3.54, p=0.57), GSTT1 (OR: 1.25, CI95%: 0.35-4.42, p=0.73), GSTP1 Ile105Val, (OR: 1.24, 95%CI: 0.47-3.28, p=0.67), CAT C262T (OR: 1.29, 95%CI: 0.48-3.44, p=0.61), GPX1 Pro198Leu (OR: 2.87, 95%CI: 0.59-14.06, p=0.19), gene polymorphisms and no association was noticed between the presence of albuminuria and the mentioned polymorphisms.

There are a group of factors involved in the development of diabetic nephropathy. The most important ones are poor glycemic control and high blood pressure. Hyperglycemia leads to oxidative stress, which leads to an excess production of reactive oxygen species (ROS) and a decrease of many cellular antioxidants [8, 20]. The oxidative lesions that appear in the lipids, proteins and DNA alter the structure and functionality of the cells. ROS act like a secondary messenger and influences both the regulation of intracellular signals and gene expression [20]. On the other hand, some recent studies failed in assessing a significant correlation between the glycemic control and the evolution of diabetic nephropathy in T2DM [21]. It was suggested that albuminuria is associated with proximal tubule injuries and loss of integrity of the glomerular filtration barrier when associated with obesity and insulin resistance. Our data showed no correlation between the body mass index (BMI) and albuminuria. Molitch et al. demonstrated in patients with type 1 diabetes mellitus (T1DM) that high HDL-cholesterol values were much less likely to have albuminuria [22]. Dyslipidemia with low HDL-cholesterol can alter the endothelial function which can lead to an increase of albumin excretion in urine. Our data shows a statistically significant association between low HDL-cholesterol level and the presence of albuminuria (p=0.02).

Due to the fact that a large number of diabetic patients develop DN without any aforementioned risk factors, scientific research was oriented towards investigating the genetic factors that lead to this complication. The hypothesis according to which the genetic factors are involved in DN pathogenesis was initially based on epidemiologic observations, which had shown no major differences in glycemic control in patients with T1DM and

DN compared to those without nephropathy and that a significant group of patients with severe, prolonged hyperglycemia did not develop nephropathy [23]. There are studies that found an association between albuminuria and cardiovascular autonomic neuropathy [24]. In our study we also found a statistically significant association between peripheral neuropathy and the presence of albuminuria ($p=0.005$).

Oxidative stress is the consequence of an imbalance between the ROS production and antioxidant capacity of the cells. The results of former studies confirm the role that the catalase plays in the renal protection mechanisms against the oxidative stress. Several polymorphisms of the CAT gene have been studied, and they were associated with DN in T1DM patients [7].

A study that investigated the role of C262T polymorphisms in the promoter region of the CAT gene failed to emphasize a high risk for microvascular complications (diabetic retinopathy and nephropathy) in patients with T1DM [25].

Panduru et al investigated the association between one of the CAT gene polymorphisms (A 21T, rs 7943316) and advanced diabetic nephropathy in patients with T1DM in Romania, but the results did not suggest that this polymorphism is associated with genetic susceptibility for DN appearance [26]. Similarly, our results failed to highlight a significant statistical correlation between the CAT C262T polymorphism and the presence of albuminuria. These results are similar to the above mentioned data.

A Pro198Leu polymorphism in the GPX1 gene has been associated with cancer [27] and peripheral diabetic neuropathy in patients with T2DM [28]. The GPX1 198Leu allele seems to be associated with genetic susceptibility for coronary atherosclerosis in patients with T2DM [29] and with peripheral diabetic neuropathy [28].

Matsuno et al proved that variant genotype of GPX1 Pro198Leu is significantly higher in diabetic patients with diabetic sensorimotor distal symmetric polyneuropathy (DSPN) compared to the ones without DSPN. The GPX1 Pro198Leu gene polymorphism seems to be involved in the development of the above mentioned microvascular complication of T2DM but plays no role in T2DM development or in diabetic autonomic neuropathy (DAN) development [30].

Panduru et al investigated the association between the GPX1 Pro200Leu gene polymorphism and susceptibility to DN in a lot of patients with T1DM from Romania and proved that this polymorphism might play a possible role in the development of this micro vascular complication [31].

Our results are not consistent with the aforementioned previous observations regarding microvascular complications in T2DM but in a Polish study there were no associations between GPX1 Pro198Leu gene polymorphism and DSPN in T2DM patients [32].

Datta et al. observed from their study that the concomitant deletion of GSTM1 and GSTT1 gene was high among DN patients as compared to data reported from general population in India [33].

A meta-analysis published in 2015 showed that GSTM1 null genotype and the risk of DN was significant in Asian population but not in Caucasians and no significant association was found between GSTT1 null genotype and GSTP1 Ile105Val genotype and DN which is similar to our results [15].

Conclusions

Our study included a relatively small number of patients and the results might not be conclusive for the general population, but it is the first study in Romania to investigate GSTM1, GSTT1 and GSTP1 single nuclear polymorphisms and their combined effect on the appearance of albuminuria in T2DM patients. To the best of our knowledge, this is the first study which investigate the relationship between the combined variant genotype of GSTM1, GSTT1 and GSTP1 Ile105Val, GPX1 Pro198Leu and CAT C262T gene polymorphisms and the effect on the appearance of albuminuria in T2DM patients.

In conclusion, the present study suggests that GSTM1, GSTT1 and GSTP1 Ile105Val, GPX1 Pro198Leu and CAT C262T gene polymorphisms are not associated with the susceptibility of the diabetic patients to develop albuminuria. Further research using increased sample size are needed in order to accomplish statistical relevance regarding these polymorphisms and the presence or absence of albuminuria.

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