ORIGINAL RESEARCH

The Role Of Adiponectin, TNF-α And Glutathione In The Pathogenesis And Evolution Of Type 1 Diabetes

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Introduction: Type 1 diabetes (T1DM) is a chronic autoimmune or idiopathic condition, featuring complex and unique interactions between proteins and enzyme systems. The purpose of the present study is to investigate the role of AdipoQ +276G>T, TNF-α-308G>A, GSTT1/GSTM1 polymorphic variants in the development of T1DM.

Materials and methods: The study is designed as a cross-sectional study, involving 72 diabetic cases and 90 controls. Genotyping was carried out according to specific protocols for the above-mentioned polymorphic variants.

Results: The G allele of AdipoQ was associated with the development of type 1 diabetes (OR 0.577, CI95% 0.336–0.802, p=0.001), similar to the GG and GA genotypes (OR 0.405, CI95% 0.156–0.654, p=0.001 and OR 0.623, CI95% 0.401–0.855, p=0.004). The G allele of TNF-α was marginally associated with the development of type 1 diabetes (OR 0.789, CI95% 0.579–0.956, p=0.005). The presence of the T1 genotype was a strong predictor for type 1 diabetes (OR 3.4, CI95% 1.433–6.243, p<0.001).

Conclusion: The results of our study suggest that G alleles of AdipoQ and TNFα act as a protective factor in T1DM, while the T1 allele for GST could be considered a risk factor for the development of Type 1 diabetes in our study group.

Keywords: adiponectin, TNF-α, glutathion, pcr-rflp, multiplex, type 1 diabetes

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune or idiopathic disease which targets the β cells of the pancreas, by way of auto-reactive T-cells, pro-inflammatory cytokines, reactive oxygen species and loss of insulin.[1,2] These changes bring forth a life-long need for insulin therapy, with important socio-economical and quality of life implications. From a genetic point of view, T1DM is characterised by complex and unique interactions between enzyme systems.

Adiponectin is a plasma protein produced by adipose tissue, with a potential role as an insulin-sensitizing agent.[3] Its gene is located on chromosome 3q26-27, a locus linked to diabetic nephropathy.[4] Low serum adiponectin concentrations (under 10 μg/L) are considered a risk factor for macrovascular complications of persistent hyperglycaemia (as encountered in both Type 1 and Type 2 diabetes).[5–7]

Glutathione is one of the major systems involved in the detoxification of xenobiotics and reactive oxygen species. Glutathione S transferase (GST) is part of that system, an enzyme which catalyses the conjugation of the toxin with glutathione. It presents in various isoforms, of which we mention the mu (M1) and theta (T1) – these often present a polymorphic homozygous deletion, resulting in a null genotype and a complete absence of enzyme activity.[1]

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Tumor necrosis factor α (TNF α) activates the RTNFα receptors, inducing the apoptosis of autoreactive T cells and the expansion of Treg cells. The α308A polymorphic variant is frequent in diabetic patients, while serum concentrations of TNFα are increased in diabetic patients.[8]

The purpose of the present research article is to offer information regarding the presented genetic variants in a Romanian pediatric population of patients diagnosed with type 1 diabetes.

Subjects And Methods
Ethics Statement
The present study has been approved by the Iuliu Hațieganu University of Medicine and Pharmacy in Cluj-Napoca, Romania, and the patients' and their legal representatives' informed consent was taken, in writing, before inclusion in the study, and was conducted in accordance with the Declaration of Helsinki.

Study Design
The study is a cross-sectional, observational case–control study. The study group includes patients diagnosed with type 1 diabetes mellitus, under or at 18 years of age, under surveillance at the First Pediatrics Clinic of the Pediatric Emergency Hospital Cluj-Napoca-this led to a total of 72 cases: 45% male patients and 55% female patients. Type 1 diabetes mellitus was diagnosed based upon one of the following criteria: Fasting (at least 8 hrs) plasma glucose ≥126 mg/dl measured on more than one occasion, a value of glycated hemoglobin (HbA1C) above 6.5% percent or random venous plasma glucose of more than 200 mg/dl, in a patient presenting classic symptoms of hyperglycemia.[9,10] The control group was comprised of patients under or at 18 years of age, admitted to the First Pediatrics Clinic of the Pediatric Emergency Hospital Cluj-Napoca for different complaints that were unrelated to diabetes. Patients diagnosed with diabetes, either type 1 or 2 diabetes mellitus, as well as pre-diabetic states or symptoms indicating diabetes, were excluded from taking part in the control group. The control group was comprised of 90 controls, with 56% males and 44% female participants.

A 10-mL peripheral blood sample was taken on a purple cap K3EDTA vacutainer, and kept at 4°C until DNA extraction was performed, using a commercially available genomic DNA extraction kit (Wizard DNA Extraction Kit, Promega Corporation). Purity and obtained DNA concentration were tested spectrophotometrically, and upon validation, genotyping was carried out.

To determine the total adipokine plasma concentration, fasting venous blood samples were drawn, without the use of anticoagulant, from which the serum was separated through centrifugation. The serum was then processed by the ELISA method for adiponectin, immunoturbidimetric method for alpha 1 antitrypsin and chemiluminescence immunoassay method for TNFα.

Genotyping Of Adiponectin, GSTM And GSTT, TNF-α Polymorphisms
Genotyping for AdipoQ 276 G>T followed a PCR-RFLP protocol, using the following primers: 5′-TCT CTC CAT GGC TGA CAG TG-3′ and 5′-AGATGC AGC AAA GCC AAA GT-3′, amplified under the following thermocycling conditions: denaturation for 10 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 30 s at 72°C, with a final elongation of 7 mins at 72°C. The amplified AdipoQ fragment was digested overnight at 37°C using 5U of Mva1269I (Fermentas MBI, Vilnius, Lithuania) and migrated through a 3% agarose gel (MetaPhor Agarose, Cambrex Bio Science Inc.), distinguishing the following possible genotypes: TT, GT, GG.

Genotyping for glutathione S transferase M1/T1 followed a multiplex PCR protocol, using three sets of primers, as follows:

5′-GAACTCCCTGAAAAGCTAAAGC-3′; 5′- GTTGG GCTCAAATATAGGGTGG- 3′and 5′-TTCCTTACTGGTCC TCACATCTC-3′; 5′- TCACCGGATCATGGCCAGCA-3′, as well as an internal amplification marker consisting of β globin, with the primer sequences: 5 ′ - C A A C T T C A T C C A C G T T C A C C - 3 ′ and 5′-GAAGAGCCAAGGACAGGTA C-3′.

100 ng of genomic DNA was amplified in 25 µl reaction mixture, comprised of: 1.5nM MgCl, 20 pmol of each primer, 200 µm of dNTPs and 0.5 units of Taq polymerase.

The mixture was amplified under the following thermocycling conditions: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 72°C and a final polymerisation step for 10 mins at 72°C. The PCR product was submitted to electrophoresis in a 2% agarose gel (MetaPhor Agarose, Cambrex Bio Science Inc). Lack of amplification signifies a null genotype.

For TNFα, genotyping followed a PCR-RFLP protocol using the following primers: 5′-TCCCCAAAAGAAAT

GGAGGCAATA-3′ and 5′-GGTTTTGAGGGCCATGAGA CGTCTGCTGGCTGGGTG-3′. The amplification conditions consisted of 12 mins at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 60°C, elongation for 1 mins at 72°C and 5 mins for a final elongation at 72°C. The amplified sequences were digested using 5 units of NcoI enzyme (Thermo Fisher Scientific Inc., MA, USA). The resulted fragments were separated on a 3% agarose gel (MetaPhor Agarose, Cambrex Bio Science Inc.); the electrophoretic analysis revealed three banding patterns, corresponding to: A1A1 wild-type homozygous genotype, A1A2 heterozygous type and A2A2 homozygous mutant genotype.

Statistical Analysis

Statistical analysis was performed by the use of SPSS for MacBook software (SPSS, Inc. Chicago, IL). Hardy–Weinberg Equilibrium was measured using the Chi-squared ($\chi2$) test. The continuous variables were presented as mean ± SD and categorical variables as percentages. The Pearson's $\chi2$ test and phi coefficient compared demographic and clinical data. Serum adiponectin and TNF-alpha concentrations were compared between subgroups using Mann–Whitney U or Student's t tests and the correlations with continuous variables were calculated by the use of Spearman or Pearson coefficients. The association between HbA1c and other serum parameters was carried out by a multivariate linear regression model. The examined allelic polymorphisms among cases and controls were tested using Fisher's exact test (OR with 95% confidence intervals). Log-linear analysis was used to determine the susceptibility of GSTM and GSTT polymorphisms for diabetes. A significant difference between groups was considered at a p-value <0.05.

Results
Demographic Study

The characteristics of type 1 diabetic subjects (n=72) regarding demographic and clinical data are presented in Table 1. The control group was composed of 90 healthy individuals with biochemical parameters and clinical data in normal limits. The serum adiponectin and TNF-alpha concentrations were not determined for the control group. There was reported a difference in age mean, but with no influence on the variant genes investigated.

Table 1 Biochemical And Demographic Parameters Of The Case Group

Parameters	Cases (n=72)	Controls (n=90)
Age mean (years)	11.93±4.27	10.19±5.08
Age of onset (years)	6.84±3.97	N/A
Male n (%)	33 (45.83)	48 (53.3)
Female n (%)	39 (54.16)	42 (46.7)
Body mass index (kg/m2)	18.83±3.36	17.32±4.06
Weight (kg)	44.89±17.4	37.00±19.8
Systolic blood pressure (mmHg)	100.2±11.85	101.5±12.9
Diastolic blood pressure (mmHg)	54.72±10.64	56.83±9.32
Cholesterol (mg/dL)	169.56±41.02	153.19±22.3
HDL (mg/dL)	54.47±11.27	53.40±9.76
Triglyceride (mg/dL)	85.32±48.5	72.43±29.0
HbA1c (%)	8.8±1.74	4.81±0.451
Adiponectin (µg/L)	14.5±4.01	N/A
TNF alfa (pg/mL)	11.18±3.55	N/A
Insulin intake (UI/kg/day)	0.95±0.27	N/A
Injections/day n (%)	4.28±0.98	N/A
Neuropathy n (%)	24 (33.33)	N/A
Nephropathy n (%)	8 (11.11)	N/A
Retinopathy, n (%)	2 (2.77)	N/A
Other complications	59 (81.94)	N/A

Note: Data are presented as mean ± SD or as a number (percentage).
Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation; HDL, high-density lipoprotein; HbA1c, glycosylated hemoglobin; TNF-alpha, tumor necrosis factor-alpha.

Analyses Of The Variant Genes, Clinical And Serum Parameters
Adiponectin

Analysis of adiponectin serum concentration was higher in men than in women in the diabetic individuals (mean±SD: 14.28±3.78 µg/L than 14.22±3.28 µg/L, $p<0.04$) and in patients diagnosed with dawn phenomenon than in other patients unrelated to gender (14.25±3.71 µg/L than 13.97 ±3.45 µg/L, $p<0.03$). Only 9 (12.5%) diabetic individuals had adiponectin serum levels <10µg/L and with minor corresponding complications related to atherosclerosis.

The serum level of adiponectin correlated positively with HbA1c ($r=0.39$, $p<0.001$), and negatively with BMI ($r=-0.34$, $p<0.01$), systolic ($r=-0.29$, $p=0.004$) and diastolic ($r=0.28$, $p=0.002$) blood pressure. Negative correlations were also found with serum cholesterol and triglyceride concentration ($r=-0.55$, $p=0.03$; $r=-0.49$, $p=0.02$, respectively); and a positive one with HDL ($r=0.8$, $p<0.001$).

Hardy–Weinberg Equilibrium was respected for all the polymorphisms studied. The genotype and allele frequency of 276G>T Adiponectin gene variant can be consulted in Table 2. Serum adiponectin concentration was also higher in GT genotype patients compared to GG and TT genotypes (GT 14.41±3.73 μg/mL, p=0.024; GG 14.21±3.69 μg/mL, p=0.025; TT 14.32±3.75 μg/mL, p=0.021). In the multivariate linear regression model, the association between HbA1c and adiponectin level remained significant after adjustment for age, sex, BMI, presence of GG, GT and GT genotypes [R^2: 0.323, beta: 0.44 (95% CI: 0.111–0.431), p<0.02].

The dominant model of Fisher's exact test for evaluating the risk for type 1 diabetes mellitus predisposition did not reveal any statistical difference for the gene variant carriers of adiponectin 276G>T between the two groups studied (P=0.012). Only the GG genotype does identify slightly increased risk for developing diabetes under the recessive model, but with no significant statistical association ($\chi2$ =2.091, OR=1.104 95% CI=0.696–1.402, P=0.06).

GSTM & GSTT

The risk associated with GSTT analyses revealed that the present genotype T+ (p<0.001) is associated with a predisposition for T1DM, conferring a 3.2-fold elevated risk (Table 3). In addition, GSTT polymorphism through the present genotype revealed an association with increased levels of HbA1c (p=0.031), but no association was shown with blood pressure, both systolic (p=0.01) and diastolic (p=0.02) when compared to the present genotype in diabetic individuals.

The genotype (M-/T+) for both groups (31.94% and 27.27%) revealed an elevated predisposition for T1DM (p=0.002), conferring a 3.651-fold elevated risk (p=0.008) (Table 4). The analyses of Log linear showed no interaction between the GSTM and GSTT combined for susceptibility to T1DM (x2=2.01, DF=1, p=0.1) or any isolated effect of GSTM (x2=1.5, DF=2, p = 0.5) and GSTT (x2 = 9.5, DF = 2, p = 0.2).

TNF-Alpha

Controls compared to cases showed a significant higher frequency of TNF-alpha GG genotype (p=0.004, OR=0.830, 95% CI 0.601–0.976) (Table 2). Regarding allele frequencies, the cases revealed a significant increase in the frequency of TNF-alpha allele G (p=0.005, OR 0.789, 95% CI 0.579–0.956). The dominant model of Fisher's exact test did not reveal any significant increased risk for diabetes regarding heterozygous and mutant genotype ($\chi2$=2.091, OR=1.197 95% CI=0.635–1.351, p=0.08; $\chi2$=3.107, OR=1.321 95% CI=1.135–1.611, p=0.06, respectively). The recessive model did not reach statistical significance at all.

In our case, TNF-alpha GA genotype and corresponding TNF-alpha level correlated positively with adiponectin serum level (r=0.44, p=0.01). The 308G/A TNF-alpha variant gene was associated with a decreased 1.056-fold risk to develop metabolic syndrome (p=0.003); correlating negatively with lipid profile (r=−0.42, p<0.02). TNF-alpha GG genotype correlated positively with HbA1c (r=0.21, p<0.01).

Discussion

Regarding the adiponectin polymorphic variant, both the GG and the GT genotypes seemed to be protective factors

Table 2 Genotype Distribution And Allele Frequency Of 276G>T Adiponectin And TNF-Alpha−308G/A In Diabetic And Control Subjects

SNP ID	Variant	Cases n(%)	Controls n(%)	OR (95% CI)	P-value
276G/TAdiponectin	GG	11 (15.27)	22 (24.44)	0.405 (0.156–0.654)	0.001
	GT	38 (52.77)	41 (45.55)	0.623 (0.401–0.855)	0.004
	TT	23 (31.94)	27 (30)	0.942 (0.806–1.128)	0.003
	GT + TT	61 (84.71)	180 (90)	0.876 (0.587–1.988)	0.009
	G allele frequency	60 (41.66)	85 (47.22)	0.577 (0.336–0.802)	0.001
	T allele frequency	84 (58.33)	95 (52.77)	0.905 (0.879–1.126)	0.005
TNF-alpha-308G/A	GG	53 (73.61)	60 (66.66)	0.830 (0.601–0.976)	0.004
	GA	17 (23.61)	30 (33.330)	1.197 (0.635–1.351)	0.08
	AA	2 (2.77)	-	1.321 (1.135–1.611)	0.06
	G allele frequency	123 (70.83)	150 (83.33)	0.789 (0.579–0.956)	0.005
	A allele frequency	21 (29.16)	30 (16.66)	1.201 (1.002–1.487)	0.07

Note: Data are presented as mean ± SD or as a number (percentage).
Abbreviations: OR, odds ratio; CI, confidence interval.

Table 3 Genotype Distribution And Frequency Of Alleles In Diabetic And Control Subjects Of GSTM, GTTM. Analysis By Chi-Square And Multiple Logistic Regression To Obtain Adjusted-Odds Ratio Values (OR) And Confidence Intervals (95% CI)

SNP ID	Variant	Cases n(%)	Controls n(%)	x2 (P-value)	OR (95% CI)	P-value
GSTM	M - M +	32 (44.44) 40 (55.55)	45 (50) 45 (50)	- 0.091 (0.103)	1 (Reference) 1.4 (0.831–1.855)	- 0.2
GSTT	T - T +	24 (33.33) 48 (66.66)	37 (41.11) 53 (58.88)	- 11.21 (0.002)	1 (Reference) 3.4 (1.433–6.243)	- <0.001

Table 4 Distribution Of Genotypic Frequencies For GSTM And GSTT In The Study Population And A Risk Analysis Of T1DM. Analysis By Chi-Square And Multiple Logistic Regression To Obtain Adjusted Odds Ratio Values (OR) And Confidence Intervals (95% CI). Significant Difference Between Groups (P<0.05)

GSTM/GTTM	Cases n (%)	Controls n (%)	x2	P-value	OR (95% CI)	P-value
M +/T +	25 (34.72)	28 (31.11)	2.71	0.045	1 (Reference)	-
M -/T +	23 (31.94)	25 (27.77)	7.33	0.008	3.651 (1.891–7.043)	0.002
M +/T -	15 (20.83)	17 (18.88)	1.22	0.21	1.675 (0.702–3.128)	0.301
M -/T -	9 (12.5)	20 (22.22)	0.89	0.34	2.265 (1.771–4.216)	0.107

against the development of T1DM; indeed, the G allele itself is a protective factor (OR 0.577, CI 95% 0.336-0.0802, p=0.001), with other polymorphisms of the AdipoQ gene having a protective effect for type 1 diabetes, however acting as a risk factor for type 2 diabetes mellitus.[4,11] Serum adiponectin concentrations are considered lowered under 10 µg/L – this has been correlated with an increased risk of cardiovascular disease as well as retinopathy in type 2 diabetes mellitus patients. However in type 1 diabetes, the level of adiponectin is above average, with increased adiponectin levels being associated with microvascular complications.[3,12,13]

The findings for GSTT1/M1 fill in some gaps left by current literature, confirming the M1 null T1 wild type genotype as a risk factor for the development of T1DM, with the T1 wild type allele being a predictive or risk factor for T1DM.[14,15] These findings come in disagreement go against some studies stating that the GSTT1 deletion is more frequent in type 1 diabetic patients and that the GSTM1 null genotype is an independent protective factor for type 1 Diabetes.[1,16,17]

For the TNFα polymorphism, the GG genotype and G allele seem to offer a minor protective effect towards type 1 diabetes, which is contradictory to the data presented by literature.[18]

The limits of the present study are the small number of subjects included in the study and the incomplete genotype-phenotype association due to the GST genotyping technique glutathione S transferase isoforms that cannot differentiate between homozygous variant genotypes and heterozygous genotype variants. This, alongside regional differences, may justify the discrepancy between data in the literature and the results of our study.

Conclusion

The G allele of AdipoQ, as well as the G allele of TNFα, seems to exhibit a protective effect on the development of type 1 diabetes. The T1 wild-type isoform of GST, as well as the M1 null T1 present genotype, is a risk factor for the development of type 1 diabetes in our population.

Disclosure

The authors report no conflicts of interest in this work.

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