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Association of the genetic polymorphism rs11640851 MT1A 80 C/A with type 2 diabetes mellitus in the Central Balkan population

Stevan Vujic[1], Jelena Milenkovic[2], Zaklina Smelcerovic[1], Tatjana Jevtovic Stoimenov[1,3], Aleksandra Klisic[4]

1. Research Center for Biomedicine, Medical Faculty, University of Niš, Serbia
2. Department Pathophysiology, Medical Faculty, University of Niš, Serbia
3. Department Biochemistry, Medical Faculty, University of Niš, Serbia
4. Center for Laboratory Diagnostics, Primary Health Care Center, Podgorica, Montenegro

Stevan Vujic, Bulevar Nemanjica 11/11, Nis, Serbia

vujicstevan@hotmail.com

+381 63 747 1749

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Type 2 diabetes mellitus (T2DM) is the most common type of diabetes and is becoming an increasingly prevalent global health issue. Polymorphisms in genes coding metallothioneins, a group of small zinc-binding proteins that participate in antioxidative protection, are believed to be involved in T2DM pathogenesis. This study aimed to investigate the potential association of the single nucleotide polymorphism (SNP) rs11640851 MT1A 80 C/A with the T2DM risk, and to determine the impact of the genotype and allelic distribution on the diabetes-related biochemical parameters. The study included 298 subjects, 112 with T2DM and 186 healthy, non-diabetic controls. The participants' fasting glycemia and HbA1c levels were measured, while the SNP in the MT1A gene was determined using the PCR-RFLP method. There were no significant differences in the genetic distribution and allele frequency between control subjects and diabetic patients ($p>0.05$). There was likewise no association between the SNP and diabetes-associated laboratory parameters, fasting serum glucose and HbA1c levels. However, 79.6% of allele C carriers had fasting glucose levels above 7 mmol/L, versus 53.3% of subjects homozygous for allele A ($p=0.005$). Although our study did not find a direct association between the MT1A genetic variants and the occurrence of T2DM, we observed an effect of the allele C on glycemic control in the patients. Further research in a larger population is needed to expand these findings and to improve the understanding of metallothionein genes and their impact on the development of T2DM.

Key words: Type 2 diabetes; Metallothionein; Zinc; Single nucleotide polymorphism

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Povezanost genetskog polimorfizma rs11640851 MT1A 80 C/A sa tip 2 dijabetes melitusom u populaciji Centralnog Balkana

Stevan Vujić[1], Jelena Milenković[2], Žaklina Šmelcerović[1], Tatjana Jevtović Stoimenov[1,3], Aleksandra Klisić[4]

1. Naučnoistraživački centar za biomedicinu, Medicinski fakultet Univerziteta u Nišu, Srbija
2. Katedra Patološka fiziologija, Medicinski fakultet Univerziteta u Nišu, Srbija
3. Katedra Biohemija, Medicinski fakultet Univerziteta u Nišu, Srbija
4. Centar za laboratorijsku dijagnostiku, Dom zdravlja, Podgorica, Crna Gora

Stevan Vujić, Bulevar Nemanjića 11/11, Niš, Srbija

vujicstevan@hotmail.com

+381 63 747 1749

Povezanost genetskog polimorfizma rs11640851 MT1A 80 C/A sa tip 2 dijabetes melitusom u populaciji Centralnog Balkana

Tip 2 dijabetes melitus (T2DM) je najčešći tip dijabetesa i postaje sve zastupljeniji globalni zdravstveni problem. Veruje se da polimorfizmi u genima koji kodiraju metalotioneine, grupu malih cink-vezujućih proteina uključenih u antioksidativnu zaštitu, učestvuju u patogenezi T2DM-a. Cilj ovog istraživanje bio je ispitavanje potencijalne povezanosti pojedinačnog polimorfizma nukleotida rs11640851 MT1A 80 C/A sa rizikom od T2DM-a, kao i utvrđivanje uticaja genotipa i raspodele alela na biohemijske parametre povezane sa dijabetesom. U istraživanju je učestvovalo 298 ispitanika, od kojih je 112 imalo T2DM, dok su 186 zdravih ispitanika bili kontrolna grupa. Ispitanicima su mereni glikemija natašte i nivo HbA1c, dok je polimorfizam u MT1A genu utvrđen pomoću PCR-RFLP metode. Nisu primećene značajne razlike u distribuciji genotipova i frekvenciji alela između kontrolne grupe i pacijenata sa dijabetesom ($p>0.05$). Takođe, nije bilo povezanosti između polimorfizma i laboratorijskih parametara povezanih sa dijabetesom, glikemije natašte i nivoa HbA1c. Međutim, 79.6% nosilaca alela C je imalo nivo glukoze iznad 7 mmol/L, u poređenju sa 53.3% ispitanika koji su homozigoti za alel A ($p=0.005$). Iako naše istraživanje nije pronašlo direktnu povezanost između genetskih varijanti MT1A gena i pojave T2DM-a, uočen je uticaj alela C na kontrolu glikemije kod bolesnika. Dalja istraživanja na većoj populaciji su neophodna kako bi se proširila ova saznanja i unapredilo razumevanje uticaja gena za metalotioneine na razvoj T2DM-a.

Ključne reči: Tip 2 dijabetes; Metalotionein; Cink; Pojedinačni polimorfizam nukleotida

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia caused by relative insulin deficiency (1). It is the most common type of diabetes by far, accounting for roughly 95% of all cases, affecting over 400 million people worldwide (2). Overall incidence of DM has doubled between 1990 and 2017, with the vast majority of cases T2DM (3). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs and systems, especially cardiovascular and peripheral nervous systems, as well as the eyes and kidneys (1).

Alterations in essential trace elements, including zinc, metabolism have been repeatedly observed in T2DM patients (4). Reduced levels of serum zinc in diabetic patients have been reported in multiple studies (5), and some linked hypozincemia to the severity of diabetes and associated complications, such as diabetic retinopathy, diabetic nephropathy and diabetic peripheral neuropathy (6-9). Earlier studies have also found that Zn ions are involved in insulin synthesis, storage, secretion, and as insulin signaling, leading some authors to propose inclusion of impaired zinc metabolism to the list of metabolic disorders in diabetes (10).

Moreover, studies have demonstrated that a family of small, cysteine-rich proteins called metallothioneins (MTs), may have an important role in Zn ion buffering and signaling, as well as in nitric oxide (NO) signaling and oxidative stress defense. Previous studies have suggested that MTs have important roles in essential metals homeostasis, defense against heavy metal poisoning and as anti-oxidative protection (11) due to their ability to scavenge reactive oxygen and nitrogen species (ROS/RNS) (12-14).

There are four main MTs isoforms in humans (15,16), two of which, MT1 and MT2, are expressed in majority of tissues, most abundantly in liver, pancreas, intestine and kidney (17).

Interest in MTs has grown over the years, as they were found to be implicated in with variety of pathological processes, from accumulation and toxicity of heavy metals (Cd, Hg, Pb), to association with multiple types of cancer, cardiovascular diseases and diabetes mellitus, and psychiatric disorders such as autism (18).

The expression levels and function of MTs are highly variable and dependent on polymorphisms present in the MT coding genes, which may contribute to the occurrence and development of various pathologies. To date, only a handful of studies have investigated the association between MT1 and MT2 gene single nucleotide polymorphisms (SNPs) and their contribution to the development of T2DM. (18-20).

One of the SNPs of particular interest in T2DM is rs11640851 *MT1A* 80 C/A, also known as *MT1A* Thr27Asn or *MT1A* +647 A/C, a polymorphism located in the coding region of *MT1A* gene that leads to an amino acid substitution. Association of this polymorphism with T2DM was suspected (21), but it was never reported before in the Central Balkan population.

This study aimed to investigate the potential association of SNP rs11640851 *MT1A* 80 C/A with the T2DM risk, and to determine the impact of the genotype and allelic distribution on the diabetes-related biochemical parameters, specifically patients' glycemia and glycated hemoglobin levels.

Materials and methods

Patients

In the study, a total of 112 patients with T2DM were recruited from the Endocrinology, Diabetes and Metabolic Diseases Clinic, University Clinical Center Niš. The patients were recruited while attending the clinic for their routine check-ups. Additionally, 186 healthy subjects were recruited as non-diabetic controls.

Biochemical analyses

Fasting serum glucose and glycated hemoglobin (HbA1c) levels were measured in all of the study participants using standard methods on automated clinical chemistry analyzer Beckman-Coulter AU680 at the Medical and Clinical Biochemistry Center, University Clinical Center Niš. Genotyping was performed in the Laboratory for Functional Genomics and Proteomics of the Scientific Research Center for Biomedicine of the Medical Faculty in Niš.

MT1A 80 C/A rs11640851 genotyping

The subject's DNA was extracted from 200 µL of whole blood, which was sampled in 3 mL EDTA tubes. A commercial DNA purification kit (Genomic DNA Purification Kit, Thermo Scientific, Lithuania) was used for DNA extraction according to the manufacturer's instructions. Genotyping of the SNP in *MT1A* gene 80 C/A rs11640851 was performed using the PCR-RFLP method adapted from Cipriano et al. 2006 (22). The primers used were: forward, 5'-CACTCAGCTGGCAGCATTTG-3' and reverse, 5'-ACTTGGCTCAGCCCCAGATT-3'. The reaction mixture consisted of 0.1 µL HotStart DNA polymerase (FIREPol DNA polymerase, Solis BioDyne, Tartu, Estonia), 2 µL FIREPol Buffer B 10x, 1.2 µL $MgCl_2$, 0.2 µL dNTP mix (20 mM of each), 0.4 µM of each primer, 1 ng/µL of DNA template, and PCR grade water was added up to a total volume of 20 µL. Amplification was performed using the following program: initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30s, primer annealing at 61°C for 45s, elongation at 72°C for 60s, and final elongation at 72°C for 7 minutes. The PCR product (187 bp) was digested using the MnlI restriction enzyme (NEB, Ipswich, MA, USA) at 37°C for 50 minutes, and the resulting fragments were resolved using vertical electrophoresis on an 8% polyacrylamide gel. The gel was then stained in ethidium bromide solution and observed under UV light. The presence of the allele A was identified by the undigested 187 bp band, while the allele C was identified by two bands of 140 and 47 bp. Heterozygous samples displayed all three bands.

Statistical analysis

The distribution of the genotypes for the polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium (HWE). The characteristics of the study group were expressed as the median and interquartile range, or the mean and standard deviation or frequency (with or without percentages). Student's t test (for normally distributed data) or the Mann–Whitney U test (non-normally distributed data) was employed for the comparison of two independent samples. ANOVA (for normally distributed data) with Tukey as Post Hoc Test and Kruskal Wallis (non-normally distributed data) with Mann-Whitney U test as Post Hoc were used to compare more than two defined groups. Chi-square (χ^2) test was used to compare data between groups,

when data were defined as categorical. All analyses were performed using IBM SPSS Statistics for Windows, v.24.0 (IBM Corp, Armonk, NY, USA) with the significance level set at $p<0.05$.

Results

General characteristics and biochemical parameters of the subjects are displayed in Table 1. Subjects with diabetes were on average of older age, had greater overall body weight, and had increased BMI ($p<0.001$). Diabetic patients had significantly increased fasting glucose levels compared to the control subjects ($p<0.001$), as well as significantly increased levels of HbA1c ($p<0.001$).

Table 1. Characteristics of the study population

	Non-Diabetics (n=186)	Diabetics (n=112)	Test (t/Z, χ^2) and significance
Sex (male/females)	30.65% / 69.35%	50.00% / 50.00%	χ^2 =11.124; p=0.001
Age (years)	60.31±9.70 60 (13)	65.12±9.19 65 (12)	t= -4.229; p<0.001
Weight (kg)	80.52±15.33 78 (20)	88.63±15.40 88 (22)	Z= -4.393; p<0.001
Body mass index (kg/m^2)	28.09±4.11 27.92 (5.68)	30.63±4.44 31.00 (5.83)	t= -4.972; p<0.001
Serum glucose level (mmol/L)	5.57±0.53 5.50 (0.70)	8.34±2.65 8.00 (2.70)	Z= -11.820; p<0.001
HBA1c (%)	5.43±0.40 5.40 (0.50)	7.60±1.71 7.00 (2.40)	Z= -13.257; p<0.001

t- Data are presented as mean±standard deviation and media (interquartile range) or frequency (%). Student – t Test; Z-Mann Whitney U Test; Chi-Square (χ^2) Test;

Of the total number of 298 subjects enrolled in the study, 279 were genotyped for MT1A 80 C/A polymorphism. Of these 180 were control subjects and 99 had diabetes.

Distribution of genotypes of *MT1A* 80 C/A in subjects is summarized in Table 2, and was in Hardy-Weinberg equilibrium in both control and diabetic groups ($p>0.05$). No significant differences in genotypes were found between the diabetes patients and controls. Minor allele

frequency (allele C) was 0.37 in the control group and 0.34 in the diabetes group, which was not a significant difference (p>0.05).

Table 2. Genotypes and alleles of MT1A in relation to diabetes presence

	MT1A AA	MT1A AC	MT1A CC	Allele A frequency	Allele C frequency
Non-diabetics (n=180)	75	78	27	0.63	0.37
Diabetics (n=99)	45	41	13	0.66	0.34
Test and significance	χ^2 =0.424; p=0.809			χ^2 =0.4453; p=0.505	

To assess the potential impact of the C allele on diabetes risk, a genetic dominant model that classified AA genotypes differently from AC and CC genotypes was used (Table 3). However, no significant difference in C allele frequency was found between the control and diabetes groups.

Table 3. Genetic dominant model of MT1A genotypes in relation to diabetes presence

	MT1A AA genotype	MT1A C allele (AC+CC genotype)	Test and significance
Non-diabetics (n=180)	75	105	χ^2 =0.374; p=0.541
Diabetics (n=99)	45	54	

Association of *MT1A* 80 C/A with the laboratory parameters of diabetes, fasting glucose and HbA1c levels, was also explored in the diabetes group (Table 4). Average glycemia in the allele C carriers was 8.68 ± 2.82 mmol/L vs. 8.15 ± 2.60 mmol/L in the genotype AA patients, which was not significant difference (p>0.05). Also, HbA1c levels were similar between allele C carriers and non-carriers (7.67 ± 1.89% vs 7.59 ± 1.67%, p>0.05).

Table 4. Serum glucose level and HbA1c with respect to MT1A genotype

	MT1A AA genotype	MT1A C allele (AC+CC genotype)	Test and significance
Fasting glucose (mmol/L)	8.15±2.60 7.20 (3.80)	8.68±2.82 8.30 (2.20)	Z=-1.409; p=0.159
HBA1c (%)	7.67±1.89 6.80 (2.90)	7.59±1.67 7.45 (2.50)	Z=-0.197; p=0.844

As the subjects in the diabetes group were all receiving anti-diabetic medication, a proportion of them did not show serum glucose and HbA1c values characteristic of diabetes, which were ≥ 7.0 mmol/L for glycemia and ≥ 6.5% for HbA1c, i.e. medication was masking the true diabetes phenotype. For this reason patients were stratified based on the aforementioned cutoff values for glycemia and HbA1c, both individually and together (Table 5). After the exclusion of subjects with regulated biochemical parameters, it was revealed that 79.6% of allele C carriers had fasting glucose levels above 7 mmol/L, versus 53.3% of subjects homozygous for allele A (p=0.005). On the other hand, patients with values of HbA1c ≥ 6.5% revealed no differences regarding the presence or absence of allele C (72.2% vs. 71.1%, respectively, p>0.05). In addition to that, among patients that displayed both glycemia and HbA1c levels above the cutoff values, 66.7% were allele C carriers, versus 44.4% allele A homozygotes (p=0.026).

Table 5 Genotype frequencies of MT1A with stratification by laboratory parameters

	MT1A AA genotype (n=45)	MT1A AC+CC genotype (n=54)	Test and significance
Fasting glucose (> 7 mmol/L)	24 (53.3%)	43 (79.6%)	χ^2 =7.759; p=0.005
HBA1c (> 6.5 %)	32 (71.1%)	39 (72.2%)	χ^2 =0.015; p=0.903
Fasting glucose (> 7 mmol/L) and HBA1c (> 6.5 %)	20 (44.4%)	36 (66.7%)	χ^2 =4.934; p=0.026

Discussion

Type 2 diabetes mellitus is a metabolic disorder characterized by hyperglycemia dominantly caused by insulin resistance. It manifests with a complex pathology involving dyslipidemia and hyperlipidemia, increase in weight due to accumulation of adipose tissue, as well as increased oxidative stress, proinflammatory state and endothelial dysfunction (1,23,24). Elevated glucose causes non-enzymatic glycation of proteins and lipids, leading to accumulation of advanced glycation end products (AGEs), which further exacerbate oxidative stress, LDL oxidation and activation of immune cells (25,26).

Oxidative stress is a crucial mechanism of pathogenesis in diabetes. Some of the typical findings in both diabetic patient and experimental animal models, are impaired functionality of antioxidative protection enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, catalase and paraoxonase (PON), as well as increased production of ROS/RNS and levels of oxidative stress markers, such as malondialdehyde (27). Excessive production of ROS/RNS worsens insulin resistance and accelerates development of the microvascular and macrovascular complications of diabetes (28). Some authors even suggested that diabetes mellitus itself be considered an oxidative stress disease (29).

Metallothioneins provide a substantial contribution to antioxidative defenses. Thanks to their high thiol (-SH) group content, metallothioneins react directly with ROS/RNS, neutralizing them similar to glutathione (GSH). In the case of •OH radicals, MTs neutralize them at a rate of approximately 340 times higher than that of GSH (13). Metallothioneins also sequester dangerous Fenton metal ions, such as Cu+ and Fe2+ (30). Additionally, MTs provide a stable availability of Zn ions, which are crucial cofactors of numerous enzymes, including SOD (31). Given the involvement of MTs in antioxidaive defenses, a number of researchers have explored the possible associations between MT alterations and various pathological conditions (32).

In this study we investigated potential association of the genetic polymorphism *MT1A* 80 C/A rs11640851, which causes amino acid substitution asparagine (allele A) to threonine (allele C) at position 27 of the MT1A polypeptide chain, where Asn variant is the wild type. We found that there were no significant differences in the genotype distribution and allelic frequency of *MT1A* 80 C/A SNP between control subjects and patients with T2DM. Previous studies conducted in central Italian population found a significant association of rs11640851 allele C with longevity in elderly women, as well as an increased T2DM occurrence with a higher risk of diabetic cardiovascular complications in the general population (21,22). With this in mind, a genetic dominant model was used to assess whether allele C was a potential risk allele in our study, but no significant differences were found between the control group and the group of patients with diabetes.

There was likewise no association between *MT1A* 80 C/A SNP and diabetes-related laboratory parameters, fasting serum glucose and HbA1c levels. Interestingly, the Italian group found an

association between the allele C and elevated fasting glucose and HbA1c levels in the group of diabetic patients with cardiovascular complications (21). Another, surprising, finding in the same study was that peripheral blood mononuclear cells of allele C carriers exhibited increased intracellular MT content, as well as reduced intracellular release of Zn ions upon NO stimulation. A more recent study in India did not find any association between *MT1A* 80 C/A and laboratory parameters of diabetes, but it did find increased frequency of allele C in the group of diabetic patients (33).

Since the subjects in the diabetes group were receiving various types and dosages of anti-diabetic medication, there was a possibility that this variation in medication, along with its varying effectiveness, could have influenced the laboratory measurements of fasting serum glucose and glycated hemoglobin A1c levels. To address this issue, we have stratified the patient group based on their glycemia and HbA1c levels by implementing cut-off values of 7 mmol/L for glycemia, and 6.5% for HbA1c. When stratified by HbA1c values alone, was no difference was observed in the genotype and allelic representation. However, there was a clear difference in serum glucose levels between the AA genotype patients and the patients carrying allele C, where 79.6% of allele C carriers had glycemia above 7 mmol/L, compared to 53.3% of the AA homozygotes. When cut-off values for both glycemia and HbA1c were implemented, the significance remained. This finding suggests that *MT1A* 80 C/A does have an impact on the level of glycemia control in diabetic patients.

The group of researchers led by Mocchegiani provided an explanation for how the presence of the *MT1A* 80 C allele influences the risk of diabetes including glycemic control in patients with type 2 diabetes. Under conditions of increased oxidative stress and pro-inflammatory signaling the expression of MT1 and MT2 genes is continuously upregulated, as these genes are induced by IL6 signaling and the presence of ROS/RNS. However, under these altered conditions, increased total Zn binding capacity, without the corresponding increase in zinc pool leads to Zn ion sequestration. Reduction in inducible Zn ion availability inside cells not only impairs the function of antioxidative protection enzymes that require Zn as a cofactor, but also disrupts zinc signaling and causes NF-κB over activation (34-37).

However, the majority of studies have found that MTs exhibit both antioxidant and anti-apoptotic properties, improving cell survival and functionality (14). Overexpression of MT protects cardiomyocites from oxidative damage, loss of contractility and apoptosis induced by high-fat diet, while the absence of MT exacerbates diabetic cardiomyopathy in a rodent model (38-42). Other studies have found that MTs have neuroprotective properties in the retinal and brain tissues against oxidative stress damage (31,43-46). Additionally, MTs have been found to help in the prevention of diabetes-induced tissue ischemia, by activation of angiogenesis through induction of HIF-1/SDF-1/VEGF pathway (47,48). It should be noted that experimental animal models are unable to accurately predict physiological and pathological processes in humans reliably, indicating that the mechanisms related to metallothioneins and their function in metabolism are still not fully understood.

Conclusion

This study did not find a direct association between genetic polymorphism in *MT1A* 80 C/A and the occurrence of type 2 diabetes mellitus. However, there was evidence of poorer glycemic control in diabetic patients in respect to the *MT1A* alleles they are carrying. A possible implication of the study is in the implementation of personalized medicine, by inclusion of genetic testing to the diagnostics, for better optimization of treatment. Further research in a larger population is needed to expand these findings and to improve the understanding of metallothionein gene polymorphisms and their impact on the occurrence and progression of diabetes.

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