Nikolac N., Šimundić A.M., Katalinić D., Topić E., Čipak A., Zjačić-Rotkvić V. (2009) ***Metabolic control in type 2 diabetes is associated with sulfonylurea receptor-1 (SUR-1) but not with KCNJ11 polymorphisms*****. Archives of Medical Research, 40 (5). pp. 387-392. ISSN 0188-4409**

http://www.elsevier.com/locate/issn/01884409

http://www.sciencedirect.com/science/journal/01884409

http://dx.doi.org/10.1016/j.arcmed.2009.06.006

Metabolic control in type 2 diabetes is associated with sulfonylurea receptor-1 (*SUR-1*) but not with *KCNJ11* polymorphisms

Running title: *SUR*-1 and *KCNJ11* polymorphisms and type 2 diabetes

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Abstract

Background: The sulfonylureas are hypoglycemic agents used for promotion of insulin secretion in type 2 diabetics. They bind to sulfonylurea receptor-1 (SUR-1), which is a functional subunit of the ATP-sensitive potassium channel (K_{ATP}). The other component of potassium channel is Kir6.2, encoded by gene *KCNJ11*. Polymorphisms in these genes may lead to modulated response to sulfonylurea therapy.

Aim: Aim of this study was to determine a relationship between SUR-1 [exon 16 (-3C/T), exon 31 (Arg1273Arg; AGG→AGA) and exon 33 (S1369A)] and *KCNJ11* (E23K) polymorphisms and following parameters of metabolic control in type 2 diabetes: fasting plasma glucose (FPG), postprandial glucose (PPG) and HbA1c in Caucasian type 2 diabetics of the European origin.

Methods: A total of 228 unrelated patients with type 2 diabetes on sulfonylurea therapy were included in the study. Genotyping of all polymorphisms was performed by PCR-RFLP method; biochemical parameters were determined using standard laboratory methods.

Results: There was no difference in FPG and PPG concentration in any of the genotype subgroups. However, diabetics with wild type C/C genotype of the SUR-1 exon 16 polymorphism had significantly lower HbA1c concentration compared to the patients with variant T/T genotype [6.9 (6.2-7.7) mmol/L *vs*. 8.1 (6.7-8.8) mmol/L; $p = 0.009$]. Also, patients with wild type G/G genotype of the SUR-1 exon 31 polymorphism had significantly higher HbA1c concentration compared to the patients with variant A/A genotype [7.8 (6.9-8.8) mmol/L *vs*. 6.3 (5.7-6.8) mmol/L; $p < 0.001$].

Conclusion: SUR-1 exon 16 and exon 31 polymorphisms are significantly associated with HbA1c concentration.

Keywords: diabetes mellitus type 2; genetic polymorphism; glucose concentration; HbA1c; sulfonylurea receptor-1

Introduction

The sulfonylureas are hypoglycemic agents used for promotion of insulin secretion in type 2 diabetics (1). They bind to sulfonylurea receptor-1 (SUR-1), which is a functional subunit of the ATP-sensitive potassium channel (K_{ATP}) located in pancreatic beta cells in islets of Langerhans. The other component of potassium channel is Kir6.2, inwardly rectifying ion channel forming a pore, encoded by gene *KCNJ11*. Mechanism of action of sulfonylureas includes binding to SUR-1 subunit of potassium channel causing its closure, opening of voltage-gated Ca^{2+} channels, increase of intracellular Ca^{2+} concentration and stimulation of insulin release from secretory granules by exocytosis. Closure of K_{ATP} is not initiated only by sulfonylurea action, but also by ATP production in glucose metabolism, indicated significant role of SUR-1 and Kir6.2, not only in diabetics under sulfonylurea therapy, but in diabetes in general (2,3,4).

The genes encoding SUR-1 and Kir6.2 are located on the chromosome 11p15.1. Numerous polymorphisms of these genes are reported in association with susceptibility to type 2 diabetes and diabetic phenotypes (5,6,7). These polymorphisms may lead to a loss of activity of potassium channel and to uncontrolled over secretion of insulin (2), as well as modulated response to sulfonylurea therapy (8,9).

In this study we have investigated three polymorphisms of *SUR-1* gene [polymorphism in intron 15 splice acceptor site (exon 16 -3C/T); silent polymorphism in exon 31 (Arg1273Arg; AG<u>G</u>→AG<u>A</u>) and missense polymorphism in exon 33 (S1369A)] and as well as missense polymorphism *KCNJ11* gene (E23K).

The aim of this study in type 2 diabetics on sulfonylurea therapy was to determine the relationship between *SUR-1* and *KCNJ11* polymorphisms and following parameters of metabolic control: i) fasting plasma glucose (FPG), ii) postprandial glucose (PPG) and iii) HbA1c concentration.

Materials and Methods

Subjects

Total of 228 unrelated patients with type 2 diabetes on sulfonylurea therapy were prospectively recruited during their regular quarterly chek-up at the Division of Endocrinology and Metabolic Diseases, Sestre Milosrdnice University Hospital in Zagreb,

from January to June 2007. All patients were Caucasians of the European origin. Exclusion criteria were: any therapeutic or lifestyle modification within the last year or any recent acute diabetic complication. Height and weight were measured for BMI calculation. Diagnosis of diabetes was made according to WHO criteria from 2006.
All patients complied with physician's recommendations regarding therapy and diet and signed informed consent for participating in the study. The study protocol was approved by the Ethical Committee of the Hospital.

Biochemical parameters

Samples for FPG and HbA1c determination were collected after overnight fast. Sodium Fluoride/Potassium Oxalate plasma was used for glucose measurement (FPG and PPG), whole blood with K_3EDTA for both, genotyping and HbA1c determination and serum for lipid concentration measurement (BD Vacutainer®, Franklin Lakes, NJ, USA).
Immediately after venipuncture, patients had a standard continental breakfast (4-5 ADA units). Sample for PPG measurement was collected two hours after the meal.
All analyses were performed immediately after the blood collection. Biochemical parameters were determined on automatic analyzer Olympus AU 2700, using original manufacturer's reagents for glucose, triglycerides, total, HDL and LDL cholesterol (Olympus, Hamburg, Germany) and Pointe Scientific, Inc reagent for HbA1c measurement (Canton, MI, USA).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using commercially available QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All polymorphisms were detected by PCR-RFLP method according to previously published protocols (5,10,11) with some modifications of PCR amplifying conditions and primer sequence. Genotyping conditions are described in Table 1. 10% of total samples were double-genotyped. Double-genotyping did not reveal any discrepancies in the result. One negative control was used per every 20 samples.

Table 1. Genotyping conditions

	Forward and reverse primers	Restriction enzyme	Restriction pattern (bp)
SUR1 exon 16 (-3C/T)	5-GCATCTGTCTGTCTGTCTTTCTGGG-3 5-GGAGCGAGGACTTGCCGC-3	HpyCH4V	C: 80 + 54 T: 134
SUR1 exon 31 (Arg1273Arg)	5-GTAGAACAGGGTCCTGTGGC-3 5-TGTCTCCAGTGACGAAGGTG-3	Bsl I	G: 132 + 65 + 52 + 1 A: 198 + 52
SUR1 exon 33 (S1369A)	5-AGGGAGAGGGGTGGGAAGAGTCCAA-3 5-ATTGGGTTGGGCCCGTGCACTGAC-3	Mwo I	T: 206 + 82 G: 206 + 41
KCNJ11 (E23K)	5-GACTCTGCAGTGAGGCCCTA-3 5-ACGTTGCAGTTGCCTTTCTT-3	Ban II	E: 150 + 32 + 28 K: 178 + 32

bp – base pairs

Statistical analysis

Normality of distributions for quantitative variables (age and biochemical parameters) was tested using Kolmogorov-Smirnov test. Normally distributed variables were presented as mean ± standard deviation, and those that were not distributed normally with median and interquartile range.

Genotype frequencies were calculated by direct counting. Difference between observed and expected genotype frequencies for calculating Hardy-Weinberg equilibrium was tested using Chi-square test.

Linkage disequilibrium analysis was done using Linkage Disequilibrium Analyzer 1.0 (Chinese National Humane Genome Center, Beijing, China).Overall differences in FPG, PPG and HbA1c concentrations according to genotype subgroups were tested by Kruskal-Wallis test. Dunn's method was used for *post hoc* testing between genotype subgroups.

P values < 0.05 were considered statistically significant. Statistical analysis was done using MedCalc® statistical software (MedCalc 9.3.9.0, Frank Schoonjans, Mariakerke, Belgium).

Results

Characteristics of study population are presented in Table 2. Both genders were represented equally, whereas most of the patients were obese. Only approximately one third of the patients had FPG and HbA1c concentrations below ADA and WHO recommended values of 7 mmol/L and 7%, respectively (12).

Table 2. Patient's characteristics

Characteristics	Patients n = 228
Age (years)	66 ± 10
Duration of diabetes (years)	9 (5-13)
Age at onset of diabetes (years)	56 ± 10
Duration of sulfonylurea therapy (years)	7 (4-12)
Male gender (n, %)	104 (45.6%)
BMI (kg/m^2)	29.74 ± 5.00
FPG (mmol/L)	8.3 (7.1-10.1)
PPG (mmol/L)	10.4 (8.0-13.2)
HbA1c (%)	7.7 ± 1.5
Triglycerides (mmol/L)	2.09 (1.38-2.70)
Total cholesterol (mmol/L)	5.4 ± 1.1
HDL cholesterol (mmol/L)	1.30 (1.10-1.50)
LDL cholesterol (mmol/L)	3.1 ± 1.0

Data are presented with mean ± standard deviation or median and interquartile range.

FPF – fasting plasma glucose; PPG – postprandial glucose

Overall distribution of polymorphisms is shown in Table 3. Genotype frequencies for all tested polymorphisms were in Hardy-Weinberg equilibrium.

Table 3. Overall distribution of polymorphisms

Polymorphism	Genotype/Allele	Total n = 228	p*
		n, %	
SUR-1 exon	C/C	45 (19.7)	
16 (-3C/T)	C/T	133 (58.3)	0.186
	T/T	50 (22.0)	
	C	223 (48.9)	
	T	233 (51.1)	
SUR-1 exon	G/G	136 (59.6)	
31	G/A	82 (36.0)	0.949
(Arg1273Arg)	A/A	10 (4.4)	
	G	354 (77.6)	
	A	102 (22.4)	
SUR-1 exon	T/T	90 (39.5)	
33 (S1369A)	T/G	97 (42.5)	0.502
	G/G	41 (18.0)	
	T	277 (60.7)	
	G	179 (39.3)	
KCNJ11	E/E	87 (38.3)	
(E23K)	E/K	103 (45.2)	0.842
	K/K	38 (16.7)	
	E	277 (60.7)	
	K	179 (39.3)	

p* for Hardy-Weinberg equilibrium, Chi-square test

SUR-1 exon 33 (S1369A) and KCNJ11 (E23K) polymorphisms were in strong linkage (R^2 = 0.9278). Linkage disequilibrium was not observed for any other polymorphism pair.

Therefore, only results for the E23K polymorphism are presented in the rest of this manuscript.

Differences in FPG, PPG and HbA1c concentrations in patient subgroups according to genotypes are shown in Table 4. There were no differences in FPG and PPG concentration in neither of genotype subgroups. However, HbA1c concentration differed significantly across SUR-1 exon 16 (-3C/T) and SUR-1 exon 31 (Arg1273Arg) genotype subgroups. Diabetics with wild type C/C genotype of the SUR-1 exon 16 polymorphism had significantly lower HbA1c concentration compared to the patients with variant T/T genotype [6.9 (6.2-7.7) mmol/L *vs*. 8.1 (6.7-8.8) mmol/L; p = 0.009]. Results were quite opposite for the SUR-1 exon 31 polymorphism: patients with wild type G/G genotype had significantly higher HbA1c concentration compared to the patients with variant A/A genotype [7.8 (6.9-8.8) mmol/L *vs*. 6.3 (5.7-6.8) mmol/L; p < 0.001].

Table 4. Differences in FPG, PPG and HbA1c concentrations in subgroups of patients according to genotypes of *SUR-1* and *KCNJ11* genes

	FPG (mmol/L)	PPG (mmol/L)	HbA1c (%)
SUR-1 exon 16 (-3C/T)			
C/C (n = 45)	8.3 (7.0-9.5)	10.2 (7.2-13.0)	6.9 (6.2-7.7)
C/T (n = 133)	8.2 (7.0-10.3)	10.2 (8.1-14.3)	7.6 (6.7-8.6)
T/T (n = 50)	8.4 (7.1-10.3)	10.5 (8.5-12.9)	8.1 (6.7-8.8)
p*	0.909	0.577	0.009
SUR-1 exon 31 (Arg1273Arg)			
G/G (n = 136)	8.4 (7.2-10.0)	10.4 (7.8-12.8)	7.8 (6.9-8.8)
G/A (n = 82)	8.3 (7.0-10.5)	10.7 (8.2-15.1)	7.1 (6.2-8.5)
A/A (n = 10)	7.6 (6.8-8.2)	9.5 (8.7-9.8)	6.3 (5.7-6.8)
p*	0.630	0.474	< 0.001
KCNJ11 (E23K)			
E/E (n = 87)	8.6 (7.2-10.4)	10.7 (8.1-14.0)	7.6 (6.4-8.5)

	FPG (mmol/L)	PPG (mmol/L)	HbA1c (%)
E/K (n = 103)	8.3 (7.0-9.8)	10.4 (7.6-13.0)	7.5 (6.5-9.0)
K/K (n = 38)	7.6 (6.9-9.7)	10.0 (8.0-11.6)	7.5 (6.8-8.3)
p*	0.143	0.675	0.824

Data are presented with median and interquartile range. p* - Kruskal-Wallis test.
FPF – fasting plasma glucose; PPG – postprandial glucose.

In order to further explore the effect of combined genotypes of SUR-1 exon 16 and exon 31 polymorphisms, haplotype analysis was performed. All of the 9 possible combinations were observed. We have divided our population into two groups: those with risk alleles, i.e. genotypes associated with poor metabolic control of HbA1c (TT/GG, TT/GA and TC/GG; genotypes for exon 16 and exon 31 polymorphisms, respectively; n = 129) and those with genotypes associated with lover HbA1c concentration (remaining six diplotypes; n = 99). Patients with risk alleles (genotypes) had significantly increased HbA1c concentration compared to the other group (8.0 ± 1.4% *vs*. 7.3 ± 1.5%; $p < 0.001$), while no difference in FPG and PPG concentration was observed.

Discussion

In our study we aimed to determine the association of *SUR-1* and *KCNJ11* polymorphisms with different parameters related to short-term metabolic control of type 2 diabetes. While we found no evidence for the association of FBG and PPG with any of the studied polymorphisms, HbA1c concentration was significantly associated with SUR-1 polymorphisms in exons 16 and 31.

Our results have confirmed previously described linkage disequilibrium between *KCNJ11* E23K and *SUR-1* exon 33 (S1369A) polymorphisms (13). All except eight of our study patients had identical genotypes: wild type alleles co-occurred together at both loci (same for the variant alleles). Hence, the concentrations of biochemical parameters across genotype subgroups were practically identical. We have presented our results only for the *KCNJ11* E23K genotype subgroup.

Literature reports on the association of *SUR-1* and *KCNJ11* gene variants with type 2 diabetes susceptibility, as well as diabetic parameters, are controversial.

Gene variant in intron/exon boundary (SUR-1 exon 16 (-3C/T)) has been widely studied. We have found variant T/T genotype of the exon 16 polymorphism to be associated with significant elevation of HbA1c concentration in patients on sulfonylurea therapy. In a French study by Mierhaege *et al*. patients on sulfonylurea therapy with at least one wild type allele (C/C and C/T genotypes) had significantly (35%) lower triglyceride concentration than the patients with the variant T/T genotype (14). This finding, similar to our results, indicates adverse association of the polymorphic variant with sulfonylurea therapy efficacy.

Several studies found association of this polymorphism and diabetes. One of the initial studies found this variant to be associated with diabetes in Caucasians (6). Similarly, in the study of Hart *et al*. on Dutch Caucasians, variant allele of the exon 16 polymorphism was more frequent in patients with diabetes compared to the healthy controls (15).

However, there are numerous publications that have yielded negative results. In study of Gloyn *et al*. authors have investigated exon 16 polymorphism distribution in subgroups of patients with mild and severe diabetes, according to FPG and BMI. They did not find any difference in frequency distribution among these subgroups (16). Also, Polish investigators failed to confirm association of this polymorphism with early failure of sulfonylurea therapy compared to patients treated with sulfonylurea despite long diabetes duration (17). Moreover, a large scale association study on 854 patients with type 2 diabetes and 1182 control subjects found no association of exon 16 -3C/T polymorphism with type 2 diabetes (OR = 1.05 (0.91-1.18); p = 0.57) (18).

Though being a silent nucleotide change, exon 31 (Arg1273Arg; AG<u>G</u>→AG<u>A</u>) polymorphism appears to be associated with diabetic phenotypes. In our study this variant was also associated with HbA1c concentration. Patients with variant A/A genotype had the lowest, with heterozygous G/A medium and with wild type G/G genotype the highest HbA1c concentration. This result is opposite to our finding on exon 16 polymorphism, since in this case, variant allele seems to have a decreasing effect on HbA1c concentration. Results of other literature reports on exon 31 polymorphism and diabetes are also very variable. In a French study by Reis FA. *et al*. variant A allele was more frequent in type 2 diabetics than in control subjects (OR = 1.54 (1.14-2.11)) (11). Authors have also investigated differences in phenotypic traits in carriers of different genotypes of exon 31 polymorphism. Though they have found A allele to be a risk factor for diabetes development, they have found that the

prevalence of hypertension was much higher in the wild type allele carriers (G/G) than in the carriers of the variant A/A genotype in obese subjects.

In a study on non-diabetic Mexican subjects, there was no difference in FPG and BMI across different genotypes, although subjects with A/A genotype had highest fasting insulin values, 2-h insulin and proinsulin to insulin ratio (19).

The exact molecular mechanism of the association of this gene variant with metabolic parameters associated with diabetes is not clear, since this silent genetic change doesn't imply the change in amminoacid sequence. It is possible that this polymorphism is in linkage disequilibrium with some other gene variant not tested in our study, which might, in some way, be associated with channel structure modification.

Recently, a mechanism of action of E23K polymorphism of the *KCNJ11* gene on diabetes development has been proposed. E23K sensitizes pancreatic K_{ATP} channels toward activatory nucleoside diphosphates, while inhibitory effect of ATP is reduced. This leads to over activity of K_{ATP} channels in the pancreatic beta cells and inhibition of insulin release, predisposing to diabetes development (20,21).

Though some literature findings support a promising role of E23K polymorphism in diabetes susceptibility and metabolic control, we have found no evidence that this polymorphism is associated with short-term diabetes outcome indicators.

Barosso *et al*. (22) have performed a large candidate gene association study on type 2 diabetes investigating 152 SNPs in 71 genes, including *KCNJ11* and *SUR-1* genes, among others. They have investigated type 2 diabetes susceptibility on 517 diabetics with matched control subjects and association with diabetic phenotypes on another population of 1,100 Caucasians with type 2 diabetes. They have found E23K *KCNJ11* polymorphism to be associated with diabetes with OR = 1.49; p = 0.033; though some other polymorphisms of this gene (3p+215, A190) showed protective influence on diabetes development (OR = 0.59 and 0.62, respectfully). This variant was also tightly linked to disease status.

In a previously mentioned paper by Gloyn AL. *et al*. (16), homozygous K/K genotype was more frequent in type 2 diabetics than in control subjects (18% *vs*. 9%; p = 0.007). No association with response to sulfonylurea therapy or with fasting plasma glucose concentration was observed. The same group of authors, few years later, performed a large meta-analysis on approximately 2,500 patients (18). The effect of E23K polymorphism on diabetes susceptibility was confirmed (OR = 1.23 (1.12-1.36)). Similar findings were replicated on several other meta-analysis by Florez (OR = 1.17; p = 0.003) (14) and Nielsen

(OR = 1.49; p < 0,001) (23). Interestingly, similarly like in the work of Gloyn *et al*., this later study also failed to associate E23K polymorphism with FPG (23).

Our research has some limitations that need to be emphasized. Though the mechanism of action of SUR-1 and Kir6.2 involves insulin pathways, due to the technical reasons, we were not able to obtain data on insulin levels. The association of selected polymorphisms and insulin level as significant factor in diabetes control needs to be further studied. Moreover, since the duration of sulfonylurea therapy was not the same for all studied patients, it could have also influenced metabolic control of diabetes.

Also, we did not examine other possible contributors of FPG, PPG and HbA1c such as diet, physical activity, comorbidity (hypertension, dyslipidemia). It is though possible that some of the studied relationships are influenced, at least partially, by these other factors. Diabetes is a multifactorial disease; well established complex genetic and environmental interactions lead to increased susceptibility to its development and progression. Metabolic changes occurring in diabetes are also of multifactorial nature; diet and physical activity being the strongest independent predictors of the phenotype. Genetic polymorphisms explored in our study are most probably not major contributors to disease metabolic parameters in diabetic patients; however our results provide clear evidence for their association with HbA1c concentration.

In conclusion, SUR-1 exon 16 and exon 31 polymorphisms are significantly associated with HbA1c concentration.

Published reports on this issue are still contradictory and we were not able to confirm all of the previous findings. This is mostly due to heterogeneity of diabetes population and small genetic effect of single gene on polygenic traits. Further studies should investigate long-term follow-up of diabetics, and determine associations of these variants with therapeutic effect and chronic diabetes complications.

Acknowledgements

This study was supported by Ministry of Science, Education and Sports, Republic of Croatia, projects #134-1340227-0200 and 134-1342428-0497.

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