Variation in *CDKAL1* gene is associated with therapeutic response to sulphonylureas

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Short title: *CDKAL1* polymorphism and sulphonylurea treatment

Summary

The aim of the present pilot pharmacogenetic study was to analyse quantitative effects of sulphonylurea treatment in addition to metformin on parameters of glycaemic control with respect to *CDKAL1* genotypes in patients with type 2 diabetes. Effect of 6-month sulphonylurea therapy on glycaemic control according to *CDKAL1* genotypes was evaluated in 101 patients with type 2 diabetes who failed to achieve glycaemic control on metformin monotherapy. *CDKAL1* rs7756992 polymorphism was determined by melting curve analysis of small amplicon following real-time PCR. After sulphonylurea treatment fasting plasma glucose (FPG) levels were significantly different (p=0.045) among three *CDKAL1* genotype groups (AA: n=49; AG: n=36; GG: n=16). In a dominant genetic model, carriers of the G-allele (AG+GG, n=52) achieved significantly lower FPG levels in comparison with patients with the AA genotype (6.90±1.08 *vs.* 7.48±1.12 mmol/l, p=0.013). Consequently, adjusted ΔFPG was significantly higher in the AG+GG compared to the AA group (1.48±1.51 *vs.* 1.02±1.33 mmol/l, p=0.022). Similar trend was observed for HbA_{1c} levels, but the difference between the genotype groups did not reach the level of statistical significance. Relatively small number of included patients is a limitation of the present study. In conclusion, our results suggest that the magnitude of FPG reduction after 6-month sulphonylurea treatment in patients with type 2 diabetes is related to the variation in *CDKAL1*.

Keywords: pharmacogenetics, sulphonylureas, *CDKAL1*, glycaemic control, type 2 diabetes

Introduction

Type 2 diabetes is a disease with significant genetic predisposition. In recent years, more than 40 genes associated with type 2 diabetes were identified by genome-wide association studies (GWAS) (Wheeler and Barroso 2011). *CDKAL1* was identified as a susceptibility gene for type 2 diabetes through five subsequent GWAS in several population cohorts of European and Asian ancestry (Steinthorsdottir *et al*. 2007, Saxena *et al.* 2007, Scott *et al*. 2007, Zeggini *et al.* 2007, Takeuchi *et al*. 2009).

CDKAL1 (cyclin-dependent kinase 5 (CDK5) regulatory subunit-associated protein 1-like 1) encodes a 65-kD protein (CDKAL-1). Although the function of CDKAL-1 is still unclear, this protein is similar to cyclin-dependent kinase 5 regulatory subunit-associated protein 1 (CDK5RAP-1), which is expressed in neuronal tissues, and inhibits CDK5 activity by binding to the CDK5 activator p35. CDK5 has been shown to blunt insulin secretion in response to glucose and to play a permissive role in the decrease of insulin gene expression, suggesting that CDKAL-1 plays a role in β-cell function by inhibiting activity of CDK5. However, at the present time, all molecular mechanisms through which CDKAL-1 modulates insulin release in pancreatic β-cells are unknown (Dehwah *et al.* 2010, Imaizumi *et al.* 2010).

The first single nucleotide polymorphism (SNP) in *CDKAL1* reported to have been associated with type 2 diabetes was rs7756992 A>G which resides in intron 5. Functional studies showed its relationship to impaired insulin secretion (Steinthorsdottir *et al*, 2007). Another polymorphism in *CDKAL1* rs7754840 was shown to be related to impaired insulin processing (Kirchhoff *et al.* 2008) and secretion (Stančáková *et al*. 2009, Palmer *et al.* 2008). Some authors suggest that pathomechanism leading to the development of type 2 diabetes in the presence of risk variants of this gene is mediated by impaired regulation of the cell cycle (Ridderstrale *et al.* 2009).

Recommended initial therapeutic interventions in type 2 diabetes include lifestyle changes and pharmacotherapy with metformin (Nathan *et al*. 2008). In patients with metformin monotherapy failure, sulphonylureas are frequently prescribed as a second line treatment (Nathan *et al*. 2008). Sulphonylureas act as insulin secretagogues through the stimulation of insulin secretion via the sulphonylurea receptor 1 (SUR-1) in pancreatic β-cells (Gribble and Reimann 2003). Considerable interindividual variation in the glucose-lowering response to sulphonylureas likely reflects variations in the β-cell secretory reserve, and may relate to variations in genes involved in regulating β-cell function. Gene variants in *ABCC8* (Zhang *et al*. 2007; Feng *et al.* 2008) and in *TCF7L2* (Pearson *et al.* 2007; Schroner *et al.* 2011) were reported to be associated with sulphonylurea effect so far.

Since genetic variations in *CDKAL1* were reported to have been associated with β-cell function (Steinthorsdottir *et al*. 2007), we hypothesised that the magnitude of sulphonylurea treatment effect might be related to the *CDKAL1* genotype. Therefore, the aim of the present pilot pharmacogenetic study was to analyse quantitative effects of sulphonylurea treatment in addition to metformin on parameters of glycaemic control with respect to *CDKAL1* genotypes in patients with type 2 diabetes.

Patients and methods

Type 2 diabetes was diagnosed according to criteria of the American Diabetes Association. The study was conducted in a university hospital setting. One hundred and one patients (50 males and 51 females, mean age 61.8±10.1 years) were recruited from three out-patient clinics. Patients were eligible for the study if they were on previous metformin monotherapy for at least 6 months, and failed to maintain HbA1c<7.0% on maximal tolerated doses of metformin at two consecutive visits within a three month period. Inclusion criteria were

HbA1c of 7.0-11.0%, age 35-70 years, and body mass index (BMI) 20-35 kg/m^2. Patients with malignancies, endocrine disorders, chronic renal failure, severe liver disease, systemic inflammatory disease and corticosteroid treatment were excluded. The ethical approval for this study was obtained from L. Pasteur University Hospital Review Board. All participating subjects gave a written consent to the study.

At the baseline visit, anthropometric data and both diabetes and metformin treatment duration were recorded. Blood samples were taken for biochemical measurements and genotyping, and sulphonylurea treatment was started with 25-50% of maximum approved dose of the specific sulphonylurea. During the interim visit(s), the doses of sulphonylureas could have been changed based on the results of blood glucose self-monitoring. Mean sulphonylurea dose prescribed on the last visit before the end of 6-month period was 47±2% of maximum approved dose for the concrete drug. Measurements of body weight, HbA1c, FPG and blood lipids were repeated after 6 months following initiation of sulphonylurea therapy.

Biochemical analyses

In all patients, peripheral venous blood samples were collected between 7-8 a.m. following an overnight 12-hour fast. Glucose was measured by glucoseoxidase method, lipids were measured by routine enzymatical methods (kits provided by ERBA-Lachema, Czech Republic), and HbA1c was measured using an immunoturbidimetric method (Roche Diagnostica, France).

Genotyping of *CDKAL1* rs7756992 (A>G)

Genomic DNA was extracted using Wizard Genomic DNA purification kit (Promega Corp., Wisconsin, USA). *CDKAL1* rs7756992 polymorphism was determined by melting analysis of small amplicon (56 bp) after the real-time PCR on LightScanner 32 instrument (Idaho

Technology Inc., Salt Lake City, USA). PCR was performed in a total volume of 10 µl containing 1x LCGreen Plus (Idaho Technology Inc.), 0.2 mM dNTPs, 3 mM $MgCl_2$, 250 ng/µl BSA, 0.9 µM each primer (forward, 5´-AATTAATATTCCCCCCTGTATTTTAGT-3´; reverse, 5´-GCTCATTGCTACATAACTGTAGAT-3´), 1 UBioThermAB polymerase with corresponding buffer (GeneCraft, Münster, Germany), and approximately 10 ng DNA. PCR conditions were as follows: initial denaturation at 95ºC for 5 min, 60 cycles at 95ºC for 10 s, 52ºC for 15 s and 72 ºC for 15 s. Amplification was immediately followed by melting analysis starting with denaturation at 95ºC for 1 min, and renaturation at 40ºC for 1 minute. Data were acquired over 60-95ºC range at the thermal transition rate of 0.05ºC/s. Genotypes were identified by different melting temperatures (homozygotes) or by change in the melting curve shape (heterozygotes), as indicated on the derivative plots using LightScanner 32 software version 1.0.0.23. Sensitivity and specificity was evaluated by testing 10 samples of each genotype (previously genotyped by PCR-RFLP method) in parallel (two replicates). All testing samples were successfully amplified, and all software-based genotype assignments corresponded with previous genotype assignments, thus, we reached 100% sensitivity and specificity. When genotyping the patient samples, melting controls (one for each genotype) were used in each run.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, USA). Relative frequencies were compared using the χ^2-test. Continuous variables are presented as mean ± standard error of mean (SE). For comparison of means either unpaired or paired Student's t-test, Wilcoxon rank test, analysis of variance (ANOVA) or ANOVA on ranks were used as appropriate. Multivariate general linear models were used to account for baseline differences and other confounding factors for the two primary

outcomes – change in FPG (ΔFPG) and HbA1c (ΔHbA1c) after treatment. All models were adjusted for age, gender, BMI, metformin dose, sulphonylurea dose and either for baseline FPG or baseline HbA1c values.

Results

In the entire group of 101 patients with type 2 diabetes 6-month sulphonylurea treatment lead to highly significant average reduction in HbA1c and fasting glycaemia by 1.1% and 1.48 mmol/l, respectively. Significant differences were recorded also in LDL cholesterol, HDL cholesterol and triglycerides levels after sulphonylurea treatment. Small and non-significant increase of body weight and BMI was observed (Table 1).

The distribution of *CDKAL1* rs7756992 genotypes followed Hardy-Weinberg equilibrium. 49 subjects were homozygous for the A-allele (AA genotype), 36 were carriers of one G-allele (AG genotype), and 16 were homozygotes for the G-allele (GG genotype).

Baseline clinical and biochemical characteristics, as well as the indices of glycaemic control after sulphonylurea treatment of three genotype groups are displayed in Table 2. No significant differences were observed in gender representation, age, weight, BMI, diabetes duration, baseline total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, FPG, and HbA1c levels among the genotype groups. After 6-month sulphonylurea treatment FPG levels were significantly different among the three groups, with the lowest mean values in patients with GG genotype ($p=0.046$). Post-hoc analysis showed that the difference in FPG after treatment was significant between AG and AA genotypes ($p=0.03$) and borderline significant between GG and AA genotypes ($p=0.05$). This result provided a rationale for pooling the subjects with AG and GG genotypes in the subsequent analysis.

Thus, in further analysis, dominant genetic model was tested in which carriers of the G-allele (AG+GG) were compared with AA genotype subjects (Table 3). After sulphonylurea therapy, patients in the AG+GG group achieved significantly lower FPG levels in comparison with patients with the AA genotype (6.90±1.08 *vs.* 7.47±1.12 mmol/l, p=0.013). Consequently, adjusted ΔFPG was significantly higher in the AG+GG compared to the AA group (1.48±1.33 *vs.* 1.02±1.51 mmol/L, p=0.022). Similar trend was observed for HbA1c levels, but the difference between the genotype groups did not reach the level of statistical significance (Table 3).

Discussion

In the present study we have shown that the carriers of the G-allele of the *CDKAL1* rs7756992 gene variant responded by significantly higher reduction in fasting glycaemia to treatment with sulphonylurea in comparison with patients homozygous for A-allele.

To our best knowledge, the present finding is the first to provide an evidence on differences in the FPG reductions between various *CDKAL1* genotype groups. Although some dysbalance in both metformin and sulphonylurea dosage was observed among the genotype groups, statistical adjustement for treatment doses of both drugs in multivariate analysis did not change the significance of association between *CDKAL1* genotype and FPG reduction with sulphonylurea treatment.

The mechanism underlying the effects of *CDKAL1* polymorphism on the therapeutic effect of sulphonylureas is unknown. Groenewoud *et al*. 2008 and Stančáková *et al.* 2008 reported that a *CDKAL1* variant (rs7754840) was related to decreased first-phase insulin secretion but not to second-phase insulin secretion during hyperglycaemic clamps and intravenous glucose tolerance tests, respectively. In an *in vitro* study, Imaizumi *et al*. found

that panceatic β-cells from mice with knocked-out *CDKAL1* gene had a reduced first-phase insulin release. Their results also indicate that CDKAL-1 controls first-phase insulin exocytosis in β-cells by facilitating ATP generation, K_{ATP} channel responsiveness and the subsequent activity of Ca^{2+} channels through pathways other than previously hypothesized CDK5-mediated regulation (Imaizumi *et al.* 2010). Since *CDKAL1* influences K_{ATP} channel responsiveness, this mechanism could explain modulation effect of sulphonylureas on glycaemic control by *CDKAL1* variant.

There are limitations in the current pilot study such as the small size of the study group. However, approximately 30% difference in ΔFPG after 6-month therapy between the homozygous carriers of the A-allele of the *CDKAL1* rs7756992 gene polymorphism and the patients with the AG+GG genotype suggests that the sample size of this study, although limited, is enough to gain understanding on the role of *CDKAL1* polymorphisms in the pharmacologic response to sulphonylureas. Furthermore, although the duration of the present study was longer compared to the previous ones (Zhang *et al.* 2007, Feng *et al.* 2008), our results reflects only the effect of sulphonylurea therapy during the first six months after initiation of this treatment. Therefore, our results cannot be automatically extrapolated beyond this period. Nevertheless, in the GoDARTS study, higher probability of sulphonylurea failure was observed up to 12 months following initiation of therapy in patients with the risk *TCF7L2* genotypes (Pearson *et al.* 2007). Therefore, *CDKAL1* genotype related differences in glucose lowering response to sulphonylureas observed in the present study might be preserved beyond six months. Further studies are needed to address this question. Importantly, investigation of a homogenous group of patients with type 2 diabetes, in whom sulphonylurea was started in a typical clinical situation after metformin monotherapy failure, represents a strength of the present study.

In conclusion, the present pilot study showed for the first time that the variation in *CDKAL1* gene is related to therapeutic response to sulphonylurea treatment. Large-scale pharmacogenetic studies are needed to confirm the associations of diabetes associated gene variants with therapeutic effects of antidiabetic drugs. Such observations might lead in the future to the development of genotype-based personalized strategies for the treatment of type 2 diabetes.

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Table 1. Baseline characteristics of the subjects and the effect of 6-month sulphonylurea treatment in addition to previous metformin monotherapy in the entire group of patients

	Before treatment	After treatment	p
Weight (kg)	84.8 ± 16.1	85.1 ± 16.1	0.28
BMI (kg/m^2)	30.3 ± 4.0	30.4 ± 4.0	0.68
FPG (mmol/l)	8.66 ± 1.41	7.18 ± 1.21	< 0.001
HbA1c (%)	8.07 ± 1.00	6.97 ± 0.60	<0.001
Cholesterol (mmol/l)	5.06 ± 1.21	4.88 ± 1.21	0.17
LDL cholesterol (mmol/l)	2.83 ± 0.91	2.6 ± 0.60	0.027
HDL cholesterol (mmol/l)	1.11 ± 0.30	1.16 ± 0.30	0.031
Triglycerides (mmol/l)	1.94 [1.79; 2.30]	1.86 [1.50; 2.10]	0.003

Data are expressed as mean ± SD or median [25^{th}, 75^{th} percentile]. p-values refer to paired Student's tests or Wilcoxon rank test. BMI, body mass index; FPG, fasting plasma glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 2. Clinical and biochemical characteristics of the subjects according to *CDKAL1* rs7756992 genotypes

	AA n=49	AG n=36	GG n=16	p
Gender (males/females)	27/22	14/22	9/7	0.28
Age (years)	63.1 ± 11.9	61.3 ± 7.20	59.8 ± 10.80	0.48
Diabetes duration (years)	2.5 ± 2.8	2.2 ± 1.8	2.7 ± 2.0	0.79
Weight (kg)	86.5 ± 16.8	82.7 ± 15.0	86.7 ± 10.4	0.51
BMI (kg/m^2)	30.7 ± 4.2	30.6 ± 4.8	30.4 ± 2.4	0.97
Metformin dose (mg)	1875 ± 325	1872 ± 392	2165 ± 334[c,d]	0.028
Sulphonylurea dose (% max.)	51.5 ± 17.4	44.4 ± 19.8	38.5 ± 17.2[e]	0.032
FPG (mmol/l) – baseline	8.70 ± 1.40	8.60 ± 1.44	8.47 ± 1.44	0.84
FPG (mmo/l) – after therapy	7.48 ± 1.12	6.94 ± 1.14[a]	6.83 ± 1.16[b]	0.045
HbA1c (%) – baseline	8.10 ± 0.91	8.17 ± 1.02	7.60 ± 0.88	0.10
HbA1c (%) – after therapy	7.07 ± 0.49	6.90 ± 0.60	6.75 ± 0.72	0.14
Total cholesterol (mmol/l)	5.20 ± 0.63	4.94 ± 1.14	5.37 ± 1.20	0.27
LDL cholesterol (mmol/l)	2.68 ± 0.42	2.86 ± 0.36	3.00 ± 0.20	0.23
HDL cholesterol (mmol/l)	1.09 ± 0.21	1.14 ± 0.36	1.10 ± 0.20	0.73
Triglycerides (mmol/l)	2.00 [1.80; 2.30]	1.90 [1.55; 2.23]	1.95 [1.41; 2.40]	0.42

Data are expressed as mean ± SD or median [25th, 75th percentile]. p-values refer to ANOVA, ANOVA on ranks or chi-squared test (for gender). Pairwise post-hoc comparisons: [a]p=0.03 vs. AA, [b]p=0.05 vs. AA; [c]p=0.012 vs. AA; [d]p=0.017 vs. AG; [e]p=0.015 vs. AA. BMI, body mass index; FPG, fasting plasma glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 3. Effect of sulphonylurea treatment on glycemic control with respect to *CDKAL1* genotypes – dominant model

	AA n=49	AG + GG n=52	p
FPG (mmol/l) – baseline	8.70 ± 1.40	8.56 ± 1.30	0.61
FPG (mmol/l) – after therapy	7.48 ± 1.12	6.90 ± 1.08	0.013
ΔFPG (mmol/l)	1.02 ± 1.33	1.48 ± 1.51	0.022*
HbA1c (%) – baseline	8.10 ± 0.91	7.99 ± 0.94	0.61
HbA1c (%) – after therapy	7.07 ± 0.49	6.85 ± 0.65	0.074
ΔHbA1c (%)	1.03 ± 0.70	1.19 ± 0.79	0.244*

Data are displayed as mean ± SD, p-values refer to unpaired Student's tests or general linear models*: ΔFPG and ΔHbA_{1c} means were adjusted in general linear models for gender, age, BMI, sulphonylurea dose, metformin dose and baseline FPG or HbA_{1c} values, respectively. FPG, fasting plasma glucose; BMI, body mass index