Analysis of the potential role of the GLUT1 and the ABCG2 transporters in type 2 diabetes

Ph.D. thesis

Anna Gelencsérné Kulin

Semmelweis University Doctoral School
Molecular Medicine Division

Supervisor: György Várady, PhD

Official reviewers: Tamás Kardon, MD, PhD
Viktória Tisza, PhD

Head of the Complex Examination Committee:
Edit Buzás, MD, DSc

Members of the Complex Examination Committee:
András Váradi, DSc
Tamás Kardon, MD, PhD

Budapest
2024

1. Introduction

Membrane proteins play a key role in biological processes, and their quantitative changes are important under various pathological conditions. In my research topic I have investigated GLUT1 and ABCG2 membrane transporters in association with type two diabetes mellitus (T2DM).

GLUT1 (*SLC2A1*) is the basal glucose transporter in various cell types, including red blood cells (RBC), as well as the endothelial cells of the blood-brain barrier and the epithelial cells of the renal tubules. Previous studies suggest that GLUT1 may have a role in the development and progression of T2DM. In case-control studies potential association was observed between some SNPs (single nucleotide polymorphism) in the *SLC2A1* gene and either T2DM or its complications.

ABCG2 is responsible for the elimination of xeno- and endobiotics from the cell and has a key role in uric acid transport. The decreased expression of ABCG2 results in significantly higher uric acid level, which is a risk factor in gout development. ABCG2-Q141K variant has a proven role in the decreased level of the protein. Since correlation between uric acid level and HbA1c was described earlier, the question arises, whether ABCG2-Q141K has a role in T2DM.

2. Objectives

The aims of my Ph.D. research were the following:

2.1. Studying the possible association between the expression level of GLUT1 membrane protein and T2DM.

2.2. Searching for genetic variants underlying the expression pattern of GLUT1.

2.3. Investigation of the exact molecular mechanism and regulatory background of the association between the identified variants and GLUT1 expression.

2.4. Examining the possible role of the SLC2A1 SNPs and the ABCG2-Q141K variant in T2DM.

3. Methods

3.1. Samples and laboratory data

The GLUT1 study was conducted involving 207 Hungarian individuals, of which 120 samples were obtained from T2DM patients, 59 samples from age-matched controls and 28 samples from healthy in-house volunteers.

The study about the ABCG2-Q141K variant was conducted involving 203 Hungarian individuals, of which 99 samples were obtained from T2DM patients and 104 samples from age-matched healthy volunteers.

3.2. Flow cytometry

First, a weak fixation process was performed to produce the RBC membranes (ghost). Then the membranes were labelled with Alexa Fluor 647 conjugated wheat germ agglutinin, followed by the specific labeling of the GLUT1 protein with rabbit monoclonal antibody. For fluorescence labelling Alexa Fluor 488-labeled goat anti-rabbit secondary antibody was used. The measurement was performed with an Attune NxT flow cytometer.

3.3. SNP selection and genetic analysis

The analyzed SNPs were selected based on sequencing results and previous literature data. The exon regions of the *SLC2A1* gene were amplified with designed primers and purified for Sanger sequencing. Genotyping experiments were carried out with TaqMan-based qPCR analysis.

3.4. Design of reporter vector constructs and cloning

To generate recombinant luciferase vector constructs, we used the basic pGL3 vector modified with a mini-TK promoter, and we analyzed inserts that contained the minor allele only for the tested SNP. The DNA fragments of interest were amplified by PCR and were inserted in the vector by restriction cleavage and ligation. The product was introduced into Dh5α ultra-competent cells, purified from the bacteria, and the sequences were verified with Sanger sequencing.

3.5. Cell culture, transfection, and treatments

For the cellular experiments, we used HEK-293T (renal derived) and HepG2 (liver derived) cells. For examining the regulatory effects of various incubation conditions, HepG2, potentially more relevant to metabolic diseases, were treated for 24 hours after transfection. When testing the effect of glucose concentration, we exchanged the medium to an FBS- and

glucose-free medium, while for insulin treatment we used a medium without FBS and a low amount of glucose, then, 6 hours before the measurements, 100 nM insulin was added.

For hypoxia treatment, cells were incubated at 37°C in a humidified CO_2 incubator with 3% oxygen level.

3.6. Dual luciferase assay

To determine the potential SNP effects, we used a Dual Luciferase Reporter Assay System kit. Luciferase reaction was measured 48 hours after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity in all experiments.

3.7. Bioinformatic analysis

In order to predict transcription factor binding sites (TFBS) affected by the examined SNPs we used the matrix scan function of the Regulatory Sequence Analysis Tools and we downloaded the newest version of the position weight matrices (PWM) of the transcription factors (TF) from the Jaspar database. This matrix contains the representation of different motifs or patterns in biological sequences. With these tools, we could predict the TF binding sites in the region around the SNPs, both for the reference and alternative alleles.

3.8. Statistical analysis

The potential association between the GLUT1 expression levels and the disease was analyzed with Mann-Whitney test. To compare genotypes and protein levels, I used the Kruskal-Wallis test with a Dunn post-hoc test. The distribution of the SNPs conformed to the Hardy-Weinberg equilibrium. I examined the SNP frequency in the control and case group with $\chi 2$ and Fisher's exact tests. The p values for the comparison of the laboratory parameters between the groups were calculated by Student's t test. To compare relative luciferase, I used the Welch's t-test. In the statistical analyses, $p<0.05$ was considered as statistically significant difference.

4. Results

4.1. GLUT1 expression and type two diabetes

In my work I measured the expression level of GLUT1 on RBCs by flow cytometry, looking for possible differences between the patients with T2DM and the healthy individuals. As a result, no difference was found between the control and case groups. However, we observed that expression levels of certain samples showed big deviation from the mean level.

4.2. Genetic background of GLUT1 expression

After considering the sequencing results and previous literature data, four SNPs (rs1385129, rs841847, rs841848, rs11537641) were selected for further analysis.

In case of rs1385129, the presence of the mutant allele showed significantly higher GLUT1 expression in RBCs, and luciferase activity in HEK-293T cells but not in HepG2 cells (**Fig.1. A/1-3**). For rs841847 the mutant allele had a decreasing effect both on GLUT1 expression and on luciferase activity (**Fig.1. B/1-3**). In case of rs841848 GLUT1 expression was higher in the presence of the mutant allele, but luciferase experiments do not confirm this result (**Fig.1. C/1-3**). Variant rs11537641 increases the GLUT1 expression level and the luciferase activity in HepG2 cells (**Fig.1. D/1-3**).

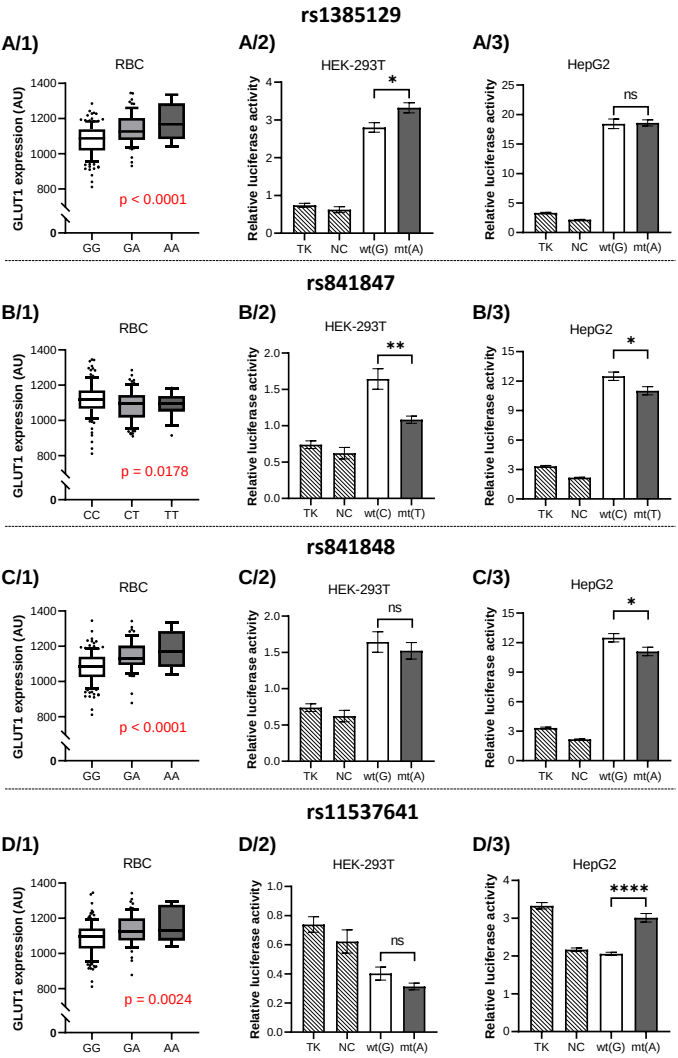

Figure 1. The effect of the four examined SNPs on GLUT1 expression and on luciferase activity.

4.3. Regulatory background of GLUT1 expression

In the following we examined some possible regulatory factors (glucose, insulin, oxygen level, TFBS) that could modulate the effects of the genetic variants.

In case of rs1385129 and rs841848, under low glucose concentrations, a difference in the effect on luciferase activity between the wild-type and mutant alleles appears. In contrast, for rs841847 and rs11537641, the significant difference between the effects of the two alleles disappears. Insulin treatment did not cause major changes, while hypoxia triggered a significant, allele-independent rise in luciferase activity.

According to our bioinformatic analysis, there are several predicted transcription factor (TF) binding sites that are altered in the presence of the tested SNPs.

4.4. SLC2A1 SNPs and ABCG2-Q141K variant in type two diabetes

Despite the previous results of the *SLC2A1* SNPs, no significant association was found with T2DM or the related laboratory parameters in our study.

When studying the SNP rs2231142 leading to the ABCG2-Q141K variant, association was also not found with T2DM.

However, we found significantly higher blood glucose and HbA1c levels in the T2DM patients carrying the ABCG2-Q141K variant (**Figure 2.**).

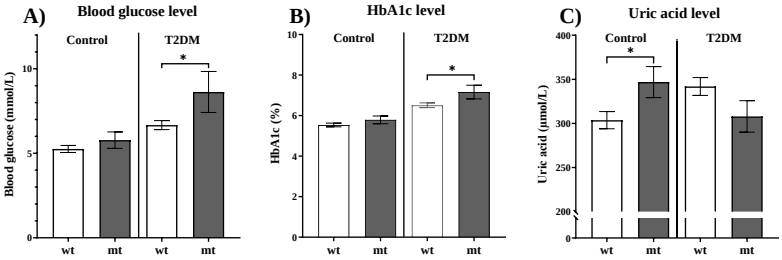

Figure 2. Blood glucose (Panel A), HbA1c (Panel B) and uric acid (Panel C) levels and the presence of the Q141K variant (hetero- and homozygotes) in the control and the T2DM groups.

5. Conclusions

5.1. GLUT1 Expression and Type 2 Diabetes

• No direct association was observed between RBC GLUT1 expression levels and type 2 diabetes mellitus (T2DM).

• GLUT1 expression levels show a relatively wide range, the background of which I found SNPs that, based on previous data, showed association with T2DM or its complications.

5.2. Genetic background of GLUT1 expression

- Four SNPs within the SLC2A1 gene (rs1385129, rs841847, rs841848, rs11537641) were examined, revealing significant effects on RBC GLUT1 expression.
- Minor alleles of rs1385129, rs841848, and rs11537641 significantly increased GLUT1 expression, while rs841847 had a decreasing effect on RBC GLUT1 expression.
- Reporter assays indicated the functional roles of these SNPs.
- The impact of rs841847 was consistent in both HEK293-T and HepG2 cell lines, while rs1385129 and rs11537641 exhibited cell-specific effects.
- Controversial results for rs841848 suggest a potential association with the GLUT1 expression pattern through another SNP in the haplotype.

5.3. Regulatory Background of GLUT1 Expression

- Metabolic factors such as glucose, insulin, and hypoxia showed variable effects on reporter expression responses.
- The effect of the genetic variants is influenced by the glucose level. In case of rs1385129 and rs841848, under low glucose concentrations a difference appears in the effect between the wild-type and mutant alleles on luciferase activity. In contrast, for rs841847 and rs11537641, the significant difference between the effects of the two alleles disappears.
- Transcription factor binding site analysis revealed important alterations in the presence of the tested SNPs, suggesting their role in the regulatory network of GLUT1 expression.

5.4. SLC2A1 SNPs and ABCG2-Q141K Variant in Type 2 Diabetes

- The three SNPs (rs1385129, rs841847, rs841848) previously described as risk factors in T2DM or its complications, were not associated with the disease in this cohort.
- The ABCG2-Q141K variant did not show direct association with T2DM but was associated with higher blood glucose and HbA1c levels in T2DM patients.
- The presence of the ABCG2-Q141K variant may affect the treatment response and contribute to long-term blood glucose alterations in T2DM patients.

6. Bibliography of the candidate's publications

6.1. Publications related to the thesis:

1. **Kulin Anna**, Kucsma Nóra, Bohár Balázs, Literáti-Nagy Botond, Korányi László, Cserepes Judit, Somogyi Anikó, Sarkadi Balázs, Szabó Edit, Várady György. Genetic Modulation of the GLUT1 Transporter Expression-Potential Relevance in Complex Diseases. Biology (Basel). 2022 Nov;11(11).
2. Szabó Edit, **Kulin Anna**, Mózner Orsolya, Korányi László, Literáti-Nagy Botond, Vitai Márta, Cserepes Judit, Sarkadi Balázs, Várady György. Potential role of the ABCG2-Q141K polymorphism in type 2 diabetes. PLoS One. 2021;16(12)e0260957.

6.2. Publications not related to the thesis:

1. Szabó Edit, **Kulin Anna**, Korányi László, Literáti-Nagy Botond, Cserepes Judit, Somogyi Anikó, Sarkadi Balázs, Várady György. Alterations in erythrocyte membrane transporter expression levels in type 2 diabetic patients. Sci Rep [Internet]. 2021 Feb 2;11(1)2765. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33531564
2. Szabó Edit, **Kulin Anna**, Jezsó Bálint, Kucsma Nóra, Sarkadi Balázs, Várady György. Selective Fluorescent

Probes for High-Throughput Functional Diagnostics of the Human Multidrug Transporter P-Glycoprotein (ABCB1). Int J Mol Sci. 2022 Sep;23(18).

3. Pálinkás Márton, Szabó Edit, **Kulin Anna**, Mózner Orsolya, Rásonyi Rita, Juhász Péter, Nagy Krisztina, Várady György, Vörös Dóra, Zámbó Boglárka, Sarkadi Balázs, Poór Gyula. Genetic polymorphisms and decreased protein expression of ABCG2 urate transporters are associated with susceptibility to gout, disease severity and renal-overload hyperuricemia. Clin Exp Med. 2022 Aug;

∑IF: 23.15