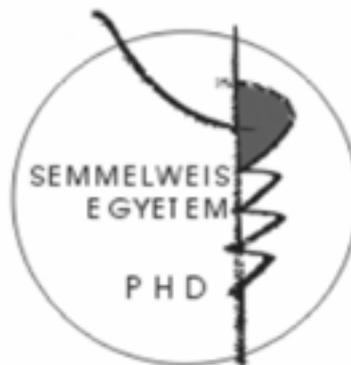


Molecular aspects of depression and diabetes mellitus

Ph.D. theses

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Introduction

Diabetes mellitus and depression are both multifactorial diseases caused by the complex interactions of genetic risk factors and environmental factors, such as sedentary lifestyle, improper nutrition, and stressful environment. Not surprisingly, both are among the most common diseases of developed countries. Recent epidemiological studies have demonstrated that depression and its associated symptoms constitute a major risk factor in the development of type 2 diabetes mellitus and may accelerate the onset of diabetic complications. On the other hand, people with diabetes are two times more likely to have depression compared to individuals without diabetes. Therefore, understanding the relationship between depression and diabetes mellitus is critical to the treatment and prevention of these diseases and their complications. The genetic components and the underlying biological mechanisms of both depression and diabetes mellitus have been studied in many different ways in the last decade.

For example, the P2RX7 gene (coding for purinergic receptor P2X₇, ligand-gated ion channel, 7) has been implicated in depression based on the results of genome wide linkage studies. The P2X₇ receptors are present on many cell types in the central nervous system (e.g., on microglia cells, astrocytes, oligodendrocytes, and neurons), however, it is still not clearly understood what kind of mechanisms these receptors are involved in the pathophysiology of depression. In our previous genetic studies a non-synonymous polymorphism (Glu460Arg) located at the carboxyl end of the protein was associated with elevated depressive and anxiety symptom score among patients with mood disorders and also in a diabetic patient population. The recent discovery of post-transcriptional regulation by microRNAs drew our attention to polymorphic variants in putative miRNA target

sites of candidate genes. Since the Glu460Arg polymorphism is located at the end of the coding region, we considered a possible linkage with single nucleotide polymorphism (SNP) in putative miRNA target sites of the 3' untranslated region of the P2RX7 gene. Based on our in silico search we have found a SNP which presumably changes the binding capability of a miRNA and is in linkage with the Gln460Arg SNP. One of the aims of this thesis was to develop a genotyping method for the miRNA binding site polymorphism in the P2RX7 gene and apply it in our genetic association study.

Another aim of this thesis was to explore the effects of diabetes mellitus on the gene expression profile of the central nervous system in order to find genes that could be associated with diabetes related neurological changes. Since studying the underlying molecular mechanisms of the increased risk for depression observed among diabetic patients is almost impossible, because of the complex nature of this phenomenon, we have approached this issue from a different point of view. We have performed a microarray-based gene expression profiling in the hippocampus, striatum, and prefrontal cortex of streptozotocin-induced and spontaneously diabetic Goto-Kakizaki rats serving as animal models for type 1 and type 2 diabetes, respectively. The lists of genes with altered expression were clustered in biochemical pathways and were also compared to literature-based candidate genes of depression or neurodegenerative diseases (e.g., Alzheimer's disease).

Aims

One of the aims of this thesis was to perform a genetic association study with a new type of functional polymorphism in the P2RX7 gene in a psychiatric and a diabetic patient population. To achieve this aim we set up the following objectives:

- 1. Perform in silico search** on miRNA binding site polymorphisms in the 3' untranslated region of P2RX7 gene that can change the binding capability of a putative miRNA
- 2. Develop genotyping methods** in order to apply in a population study of the putative miRNA binding site polymorphisms
- 3. Perform a genetic association study** of the putative miRNA binding site polymorphisms in both patient groups in order to clarify the P2RX7 genetic effects

The other aim of this thesis was to study the molecular effects of diabetes mellitus on brain functions. To fulfill this aim we performed a whole genome expression profiling in animal models of diabetes mellitus. Our main questions were:

- 4. Is there any significant change in the gene expression profile of the brain in type 1 and type 2 diabetes animal model** compared to the control?
- 5. Which brain regions** are the most affected by these changes?
- 6. Which biochemical pathways** are involved by these gene expression changes?

Methods

Participants of the genetic association study

The symptoms of depression and anxiety were assessed by the Hospital Anxiety and Depression Scale (HADS). The two scales of the HADS questionnaire consists of 7–7 questions. The psychiatric patients (N=152) were selected from the inpatients at the Department of Psychiatry, Kútvölgyi Clinical Centre. Diagnosis of major depressive disorder (N=95) and bipolar disorder (N=57) was based on DSM-IV criteria. Diabetic patients (N=218) were recruited from the inpatient and outpatient services of the 2nd Department of Internal Medicine at the Semmelweis University. Healthy control subjects without any psychiatric history and diabetic symptoms were recruited at the Institute of Psychology, Eotvos Lorand University. The study design was approved by the Local Ethics Committee (TUKEB). Every participants provided written informed consent for their participation.

Genotyping the polymorphism located in the 3' untranslated region of the P2RX7 gene by PCR RFLP

Identification of the polymorphic miRNA binding site in the 3' untranslated region of the P2RX7 gene was done by in silico data analyses using the PolymiRTS and the Patrocles databases. The genotyping process of the rs1653625 SNP included the amplification step by PCR and a digestion step by Eco24I restriction endonuclease. The DNA fragments were analyzed by two different methods. Submarine horizontal agarose gel electrophoresis was supplemented with the QIAxcel multi-capillary electrophoresis system. To assure the accuracy of the genotyping technique, nine samples (3-3 samples from the three genotype groups) were selected for direct PCR sequencing.

Statistical analyses of the P2RX7 genetic association study

The SPSS program for Windows (17.0 version) was used for the dimensional association study. Multivariate analyses of covariance

(MANCOVA) were performed to test the effects of genetic factors on the HADS subscales using age and sex as covariates. The linkage disequilibrium between the Gln460Arg SNP located in the P2RX7 coding region and the rs1653625 SNP located in the 3' untranslated region was calculated by the Haploview program. The effect of the haplotypes on the HADS subscales were analyzed by the THESIAS program.

Animal models for diabetes mellitus

The gene expression experiments were performed on ten-week old male rats. The control group consisted of age- and body mass matched inbred white Wistar rats. Streptozotocin-treated Wistar rats were used as model animals for hyperglycemia caused by type 1 diabetes, and Goto-Kakizaki rats served as the polygenic, non-obese model of type 2 diabetes. Wistar rats at 6 weeks of age were injected with 65 mg/body mass kg streptozotocin intravenously. Nine animals from each group were anaesthetized and killed by decapitation at 10 weeks of age. The brain was removed and the striatum, hippocampus, and prefrontal cortex were dissected.

RNA isolation and microarray hybridization

Brain samples from 3-3 identically treated animals were pooled together for RNA extraction. The RNA integrity and purity were checked by NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer. After reverse transcription and labeling with Cy3 dye the hybridization was performed on Agilent's Rat Whole Genome Custom 4x44K Arrays.

Microarray data analysis

The expression data analysis was performed by the Gene Spring GX software version 7.3. For normalization, the samples were grouped together according to the brain regions. Diabetic samples were normalized to the median of the control samples of each group.

Following normalization, technical data screening was applied as quality control. After the normalization and screening procedures statistical analysis was performed to select probes with at least twofold expression changes in type 1 or type 2 diabetic animals compared to Wistar controls using the Welch's t-test supplemented with the Benjamini-Hochberg multiple correction test with a $p=0.05$ cutoff. The Gene Ontology database was used to identify genes by clustering them in relevant biochemical pathways.

Microarray data validation by Real Time PCR

Genes that fulfilled the criteria of technical, statistical and pathway analyses were validated by the quantitative real time PCR-based TaqMan Low Density Array (TLDA) system, according to the manufacturer's protocol. Relative gene expression data were obtained using the $2^{-\Delta\Delta CT}$ method. Three genes were selected as internal controls for normalization of qRT-PCR data: Hdac3, Tbp, and B2m. Finally, the Mann-Whitney test ($p<0.01$) was used for statistical analysis of the qRT-PCR data.

Results

1. We conducted an in silico search for polymorphisms in putative miRNA target sites in the 3' untranslated region of the P2RX7 gene using the PolymiRTS and Patrocles databases. A total of three SNPs which were located in the essential seed region of the miRNA target site and created or deleted a putative miRNA target site were found in the 3' untranslated region, but only one (**rs1653625**) was identified in both databases. In addition, only the rs1653625 had a minor allele frequency higher than 10%; therefore, we selected this SNP for our genetic association study.
2. For the genetic analysis of the most frequent miRNA binding site polymorphism in the P2RX7 gene we have successfully developed a **new restriction fragment length polymorphism based method**. The sequence surrounding the rs1653625 A/C SNP was found to be problematic, hindering the applicability of classical genotyping techniques. Therefore, we applied a primer with mismatches close to the SNP of interest to create an allele-specific cleavage site for the restriction enzyme Eco24I. As a result of the introduced mismatches, the PCR product was cleaved by the enzyme, yielding a 157 bp and a 19 bp fragment in the presence of the C-allele. In case of the A-allele, the restriction site was not present and the PCR product was not cleaved, resulting in a single, 176 bp long fragment. The separation of the 176 bp and the 157 bp fragments was sufficient for reliable genotyping.
3. **Significant association** was detected between the A-allele of the rs1653625 and the severity of depression and anxiety symptoms both in the psychiatric and the diabetic patient groups. We showed that this miRNA binding site SNP was in **linkage** with a previously studied non-synonymous SNP (Gln460Arg where G=Arg allele is considered as the risk factor). Our haplotype analysis indicated that

the A-allele of the rs1653625 increased the severity scores even in combination with the A-allele of Gln460Arg, but the highest scores were estimated in the haplotype group consisting of both risk alleles (i.e., the G-allele of Gln460Arg and the A-allele of rs1653625) at both depression and anxiety symptoms.

4. As a result of our whole genome expression microarray assay we demonstrated **significant differences in gene expression patterns of brain regions in type 2 diabetic rats** vs. control animals. The genetically selected, spontaneously diabetic Goto-Kakizaki rats exhibited profound gene expression changes (416 genes with altered expression level). On the other hand, we could hardly detect any expression changes in the streptozotocin-induced diabetic animal model on the whole genome level.
5. Detailed analysis of the gene expression data of Goto-Kakizaki rats demonstrated many changes in the **hippocampus** (266 genes) and **prefrontal cortex** (147 genes) compared to Wistar control rats. The majority of the changes were region specific (hippocampus: 180 genes, prefrontal cortex: 61 genes), whereas 83 genes with altered expression were found in both brain regions. In contrast, only 3 genes with altered expression were identified in the striatum, and these genes were found to have expression changes in the other two regions as well.
6. Finally, pathway analysis revealed that most genes with altered expression patterns in the hippocampus are involved in **oxidative stress, DNA damage signaling, immune processes, cell cycle regulation, development of the central nervous system, lipid metabolism**, as well as in the **regulation of feeding behavior**. Regarding the prefrontal cortex, perturbed expression of a set of **neurotransmission** and **lipid metabolism** related genes has been unveiled. These findings are consistent with the present literature of

type 2 diabetes (e.g., the oxidative stress, insulin secretion, and feeding behavior pathways) and of psychiatric disorders (circadian clock, synaptic transmission, neurotransmitter uptake pathways) or neurodegenerative diseases (apoptosis, acetylcholine metabolism, Ca^{2+} homeostasis pathways). We could also directly associate some of the genes with the above mentioned diseases. For instance, the CHI3L (chitinase 3-like 1) gene which showed increased expression in the hippocampus has been indicated as a novel, obesity-independent candidate gene in type 2 diabetes. As another example, the synuclein gamma gene (also with increased expression change in the hippocampus) has a well known role in the development of Parkinson disease. Showing up-regulation in the prefrontal cortex, the protein kinase C gamma and epsilon might potentially play a pathophysiological role in brain dysfunction. For instance, the neuron-specific gamma isoform of protein kinase C (Prkcc) has been implied in the regulation of learning and memory formation, whereas the epsilon isoform of PKC (Prkce) is involved in the pathomechanism of drug dependence and addiction. Regarding both regions, **galanin** and its receptor (GALR2) could have high importance in our study. Galanin, which is an inhibitory neuropeptide, had elevated levels in the hippocampus, therefore the over-expression of galanin can elicit cognitive impairment, enhanced food intake and depression. These symptoms highly overlap with the manifestations of cerebral insulin deficiency. It is tempting to speculate that the development of symptoms of impaired cerebral insulin signaling in type 2 diabetes might be mediated partly by the up-regulation of galanin expression in the hippocampus. Moreover, over-expression of galanin might also be involved in the progression of Alzheimer's disease. Finally, we could detect some genes that could provide a link between diabetes mellitus and neurodegeneration (the so-called type 3 diabetes). Beside the already mentioned synuclein gamma, the uncoupling

protein 2 (UCP2), the ABC-transporter ABCA1, and the cell surface antigen CD47 should be mentioned in this context.

Conclusions

The results of the latest epidemiological studies show that depression is twice as frequent amongst diabetic patients compared to the control population. In this work I present our genetic analysis of a new candidate gene of depression and I make an attempt to highlight the possible pathomechanisms of the comorbidity of depression and diabetes mellitus from a different point of view.

In our previous genetic studies, significant association was found between a non-synonymous polymorphism in the last exon of the P2RX7 gene (Gln460Arg) and depression severity among patients with diabetes mellitus and among patients with major or bipolar depression. According to our hypothesis, it might be possible that a SNP in the 3' non-coding region, that influences microRNA binding, is responsible for the above mentioned genetic effect and not the exon polymorphism located at the end of the coding sequence. Therefore, we searched for polymorphisms in putative miRNA target sites in the 3' untranslated region of the P2RX7 gene. However, the identified SNP (rs1653625) subsided in a methodologically problematic region of the P2RX7 3' region, therefore, we developed a method based on restriction fragment length analysis in order to genotype this polymorphism. We successfully used this genotyping method to perform the genetic association analysis, which indicated an association of the studied miRNA binding site polymorphism with depression severity.

The second part of my thesis aims to study the central nervous system complications of diabetes mellitus. We aimed to study the molecular background of this issue with a generalized approach: we assayed gene expression changes in specific brain regions of a rat model of diabetes mellitus. After evaluating the whole genome expression data, we could detect significant changes in the expression profile of specific brain regions of type 2 diabetes mellitus model compared to the controls. On the one hand, the changes detected in the

hippocampus and the prefrontal cortex support the molecular background of the so-called type 3 diabetes. On the other hand, we managed to identify new genes in the pathomechanisms of diabetes mellitus. The pathway analyses showed that genes with altered expression level were connected to the lipid metabolism, signal transduction processes, apoptosis signaling pathways, and neurotransmission. All of these pathways can be related to the previously described discrepancies in the brain due to diabetic complications. Among the genes showing altered expression, galanin (an inhibitory neuropeptide) seems to be of utmost importance. Galanin showed up-regulation in the hippocampus of our type 2 diabetes model, and it is known that the overproduction of galanin leads to cognitive deficit, it also increases nutrient intake and causes depressive symptoms. These symptoms highly overlap with the symptoms described in the absence of insulin in the brain. Therefore, our results prove that type 2 diabetes mellitus can affect the brain on a molecular level. As far as we know, our expression assay was the first one that analyzed whole genome expression profile changes in specific brain regions of diabetes mellitus animal models.

Publications

Publications included in the thesis:

1. **Abdul-Rahman O**, Sasvari-Szekely M, Szekely A, Faludi G, Guttman A, Nemoda Z. Analysis of a polymorphic microRNA target site in the purinergic receptor P2RX7 gene. *Electrophoresis*. 2010; 31(11):1790-5. (IF.: 3,509)
2. Keszler Gergely, **Abdul-Rahman Omar**, Rosta Klára, Somogyi Anikó és Sasvári-Székely Mária. A galanin neuropeptid agyi expresszióváltozása 2-es típusú diabétesz patkánymodelljén. *Magyar Belorvosi Archivum* 2010; 63(2):87-90
3. **Abdul-Rahman O**, Sasvari-Szekely M, Ver A, Rosta K, Szasz BK, Kereszturi E, Keszler G. Altered gene expression profiles in the hippocampus and prefrontal cortex of type 2 diabetic rats. *BMC Genomics*. 2012; 13:81. (IF.: 4,07)

Further publications:

1. Than NG, **Abdul-Rahman O**, Magenheim R, Nagy B, Fule T, Hargitai B, Sammar M, Hupuczi P, Tarca AL, Szabo G, Kovalszky I, Meiri H, Sziller I, Rigo J Jr, Romero R, Papp Z. Placental protein 13 (galectin-13) has decreased placental expression but increased shedding and maternal serum concentrations in patients presenting with preterm pre-eclampsia and HELLP syndrome. *Virchows Arch*. 2008; 453(4):387-400. (IF.: 2,082)
2. Nagy G, Ronai Z, Somogyi A, Sasvari-Szekely M, **Abdul-Rahman O**, Mate A, Varga T, Nemoda Z. P2RX7 Gln460Arg polymorphism is associated with depression among diabetic patients. *Prog Neuropsychopharmacol Biol Psychiatry*. 2008; 32(8):1884-8. (IF.: 2,638)

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