OTKA_KH_125570

Final Report

Identification of drug targets for ischemic heart disease and its comorbidities by bioinformatic target prediction based on transcriptomic data followed by experimental validaton of predicted targets

1. Background

There is no available cardioprotective pharmaceutical agent on the market that could reduce the infarct size, mainly because of the hypothesis-driven, biased methodology of drug target identification and the lack of the application of comorbidty models during the drug development process. Thus our aim was to overcome the limitations of the standard hypothesis driven approaches by the network theoretical analysis of complete microRNA (miRNA) and messenger RNA (mRNA) expression profiles assessed from various combined cardioprotection and comorbidity models. Based on the expression profiles here we predicted potential molecular drug targets by *in silico* methods and experimentally validated some predicted targets.

2. Aims

2.1 Application of high-throughput transcriptome assays to describe the transcriptome profile changes at miRNA and mRNA levels in conditions of ischemia-reperfusion injury, ischemic conditioning and different comorbidities.
2.2 Based on the transcriptomic changes, identification of key regulatory points (potential drug targets) by bioinformatic tools (e.g. network analysis)
2.3 Validation of the predicted targets at transcriptomic and protein levels.

3. Experimental approach

Tissue samples for the experiments in this project originated from NVKP_16-1-2016-0017, OTKA_K 109737, Austrian-Hungarian Action Scholarship (88öu1), EFSD grant and international collaborations. Optimization of plasma and saliva sample collection and miRNA sequencing was performed. Previously validated miRNAtarget™ software was used for the network theoretic analysis of omics datasets and the unbiased prediction of most likely mRNA targets. Furthermore, network layout software was validated for the better visualization of networks created during the analysis of the omics datasets.

3.1 Hypercholesterolemia comorbidity model

To induce hypercholesterolemia Wistar rats were fed for 12 weeks with standard rat chow supplemented with 2% cholesterol and 0.25% cholate. For miRNA microarray analyses rat hearts were snap frozen in liquid nitrogen and stored at – 80 °C [1].
In the present study predicted miRNA-target interaction network consisting of nodes of miRNAs and their targets and edges representing the interactions, was constructed by miRNAtarget™ based on the global miRNA expression profile. EntOptLayout plugin (version 2.1) for the Cytoscape (version 3.6) network visualization and analysis framework was used to visualize the resulting miRNA-target interaction network [2]. Target validation with qPCR, Western blot and luciferase assay was performed.

3.2 Sensory neuropathy comorbidity model

To induce sensory neuropathy capsaicin was administered subcutaneously to the Wistar rats on consecutive days at increasing doses of 10, 30, and 50 mg/kg, respectively under isoflurane anaesthesia [3]. miRNA microarray analysis of heart samples was performed.
Similarly to the bioinformatics workflow applied in the hypercholesterolemia comorbidity model (section 3.1) previously measured miRNA expression dataset was analyzed and the most likely targets of differentially expressed miRNAs were predicted by miRNAtarget™. The resulting miRNA-target network was visualized by the EntOptLayout software [2]. Target validation with qPCR was performed.

3.3 Prediabetes comorbidity model
To induce prediabetes Long-Evans rats were fed with high-fat (40%) chow for 21 weeks and treated with a single low dose (20 mg/kg) of streptozotocin at week 4. At week 21 of the diet, animals were anesthetized with pentobarbital (60 mg/kg, i.p.), then hearts were excised, shortly perfused with oxygenated Krebs-Henseleit buffer in Langendorff mode and stored at -80°C in RNAlater [4]. Small RNA-sequencing of left ventricle (LV) samples was performed.
Following sequencing adapter trimming and filtering of raw reads the alignment to the rat reference genome and summarization of the reads were conducted [5] [6] [7]. Differential expression analysis was performed by the EdgeR [8] Bioconductor package.

Expression changes of predicted miRNA-targets caused by miRNA driven post-transcriptional regulation was estimated by the miRNAtarget™ software (https://mirnatarget.com; Pharmahungary, Szeged, Hungary), which was described in detail and was validated in related studies as detailed above [1, 3]. In the resulting network miRNAs and their targets were represented by nodes and were connected by edges of predicted interactions. After assigning positive or negative weights (1 or -1) to edges based on the up- or downregulation of the interacting miRNA respectively, node strength was calculated for each target node by summing the incoming edge weights. Optimal network visualization was obtained by the EntOptLayout software. Validation of the predicted targets with qPCR was performed.

3.4 Obesity comorbidity model
To induce obesity Long-Evans rats were fed with 20 % fat and 15 % sucrose-enriched diet for 25 weeks. A group of rats received 0.25 mg/kg selegiline ip. injection once daily from the 16th weeks of diet. For transcriptomic analyses at week 25 LV samples was collected and placed in RNAlater solution[9] . Total RNA from heart tissue was extracted and RNA concentration was measured with Qubit HS RNA kit. RNA integrity values were determined with Bioanalyser. Small RNA library construction and small RNA sequencing was performed by a subcontractor. Both raw sequencing data analysis and miRNA target prediction were performed as described 3.3 section using the miRNAtarget™ and EntOptLayout softwares.

After total RNA isolation, cDNA was synthesized from total RNA. Primer pairs were designed against sequences of intron-spanning exons by Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and tested to avoid primer dimers, non-specific amplification, and self-priming by this software. To detect the transcript levels of the primers PCR was performed. Specificity of the PCR products was confirmed by verification of the amplicon length on 2 % agarose gels stained by gel red.

3.5 Myocardial infarction model
Here we used cardiac tissues samples generated in our previous extensive study in closed chest domestic pigs [10]. Farmpigs were subjected to sham operation (Sham), ischemia/reperfusion (I/R) or postconditioning (IPostC) by inflations and deflations of a balloon catheter in the

anterior descending branch of the left coronary artery. After 3 hours or 72 hours of reperfusion, tissue samples were collected from the left ventricles. Immunoblotting analysis in whole tissue lysates from ischemic and non-ischemic zone was performed to determine signal alterations can be responsible for the myocardial edema reduction in IPostC group.

3.6 Rofecoxib comedication model

Male Wistar rats were treated with rofecoxib (5.12 mg kg^{-1}/day) or vehicle for 28 days. At day 28 animals were anesthetized with pentobarbital (60 mg/kg, i.p.), then hearts were excised, shortly perfused with oxygenated Krebs-Henseleit buffer in Langendorff mode and stored at -80°C. Total RNA from heart tissue was extracted. After RNA isolation quality check of the samples was performed using Qubit HS RNA kit and Bioanalyser. RNA library construction and mRNA sequencing was performed by a subcontractor. After adapter trimming and quality filtering (Phred score >30) of raw reads quality control analysis were performed with the use of FastQC (version v0.11.7).

3.7 Saliva, plasma miRNA profile analysis

miRNA expression profile is a potential new biomarker in several diseases. Saliva contains miRNAs and other short RNAs [11]. Several methods are available in the literature for stimulated saliva collection [12], however, there is no data about repeated collection of stimulated saliva from the same animal. Efficiency of RNA isolation from saliva and plasma is low, therefore protocol optimization in necessary. We performed the optimalisation of stimulated saliva collection from rats and we collected plasma and saliva samples. RNA isolation, library preparation and smallRNA sequencing was performed on rat plasma and saliva samples. Adapter trimmed, quality (Phred score >30) and length (>10 nt) filtered sequencing reads from our rat plasma and saliva samples were subjected to quality control analysis by FastQC (version v0.11.7) mostly focusing on the read length distributions. Raw sequencing reads from previous studies available on NCBI Sequence Read Archive were also analyzed by the same workflow and compared to the quality control parameters of our rat samples.

3.8 Relative Entropy Optimization based network visualization software

Expression changes of targets in the complex post-transcriptional regulatory network of miRNAs could be only predicted precisely, if we consider the resulting effect of each microRNA. This goal can be achieved with the use of network theoretical approaches by constructing miRNA-target interaction networks. Complex networks generated during this process however are hard to be visualized with the currently available network layout algorithms in a way that allows the identification of key mediators and functional modules in the network. To overcome this issue a new software tool called EntOptLayout was implemented for the more effective visualization of large networks modelling relations and interactions in various fields including biological pathways.

4.Results

4.1 Effect of hypercholesterolemia on cardiac gene expression profile

Forty-seven upregulated and ten downregulated miRNAs were found in hypercholesterolemic rat hearts compared to the normocholesterolemic samples. 3 mRNAs (beta-2 adrenergic receptor [Adrb2], calcineurin B type 1 [Ppp3r1] and calcium/calmodulin-dependent serine protein kinase [Cask]) were validated using qRT-PCR and Western blot. In hypercholesterolemic hearts, expression of Adrb2 mRNA was significantly decreased. ADRB2 and PPP3R1 protein were significantly downregulated in hypercholesterolemic hearts. With luciferase reporter assay a direct interaction of Adrb2 with upregulated miRNAs was demonstrated.

We published these results in an international peer-reviewed journal [1].

4.2 Effect of sensory neuropathy on cardiac gene expression profile

Out of 711 known miRNAs measured by miRNA microarray, the expression of 257 miRNAs was detected in the heart. As compared to vehicle-treated hearts, miR-344b, miR-466b, miR-98, let-7a, miR-1, miR-206, and miR-34b were downregulated, while miR-181a was upregulated as validated also by quantitative real time polymerase chain reaction (qRT-PCR). By an in silico network analysis, we identified common mRNA targets (insulin-like growth factor 1 (IGF-1), solute carrier family 2 facilitated glucose transporter member 12 (SLC2a-12), eukaryotic translation initiation factor 4e (EIF-4e), and Unc-51 like autophagy activating kinase 2 (ULK-2) targeted by at least three altered miRNAs. Predicted upregulation of these mRNA targets were validated by qRT-PCR.

We published these results in an international peer-reviewed journal [3].

4.3 Effect of prediabetes on cardiac gene expression profile

Using small RNA-sequencing analysis the expression of 356 different miRNAs was detectable in LV samples out of the 752 mature miRNA sequences available in the reference annotation. As compared to the control group 2 miRNAs were significantly upregulated while 3 miRNAs showed significant downregulation in the prediabetic group.

By in silico miRNA target analysis 445 mRNAs were predicted to be regulated by the 5 significantly differentially expressed miRNAs. 5 targets were selected for experimental validation based on Gene Ontology analysis. Out of these 5 targets, downregulation of 3 mRNAs i.e. Juxtaposed with another zinc finger protein 1 (Jazf1), RAP2C, member of RAS oncogene family (Rap2c), and Zinc finger with KRAB and SCAN domains 1 (Zkscan1) was validated.

Based on these results we submitted a manuscript in an international peer-reviewed journal.

4.4 Effect of obesity on cardiac gene expression profile

87 miRNAs were detected to be differentially expressed if only p-values are considered, out of which 11 were chosen for miRNA-target interaction analysis based on their expression pattern among the experimental groups. For these 11 miRNAs 921 targets were predicted and considering their outstanding network topological parameters 12 targets were selected for experimental validation. After total RNA isolation, cDNA was synthesized from total RNA and primer pairs were designed. 9 primers were successfully designed and the other 3 primers need to be redesigned.

We plan to publish these results in early 2020.

4.5 Effect of myocardial infarction on gene expression profile

IPostC significantly reduced edema and microvascular obstruction compared to I/R control after 72 h. Increased gene expression of CaMKIIγ on the mRNA level in I IPostC vs I/R setting was measured by RNA-sequencing.

On the other hand, comparing to Sham or I/R group, protein expression of p-TCaMKII and total CaMKIIγ/δ isoforms was not altered by IPostC in both ischemic or nonischemic parts of conditioned hearts after 3 h or 72 h since reperfusion. Expression of HIF1α, a major hypoxic and pro-angiogenic transcription factor, followed this pattern and did not significantly change due to IPostC in all analyzed groups. However, IPostC increased pSer473-Akt in non-ischemic tissue after 3 hours compared to same tissue type in I/R group, but these changes were normalized after 72 hours. Additionally, in time-dependent manner, expression of pSer473-Akt significantly increased in both I/R and IPostC non-ischemic tissue after 72 hours when

compared to their respective 3 hours controls. Autophagy showed time dependent activation in the 3day samples based on pThr172-AMPK and pSer555-ULK1 phosphorylation, but IPostC does not seem to have significant effects. Other hand LC3-I conversion to LC3-II was blocked at 3 day. Other autophagy markers were also assessed (pSer-mTOR/mTOR, beclin1, p-757-ULK1/ULK1) without significant changes with time or between groups. Results of miRNA high trough-put PCR were analyzed to identify CaMKIIγ isoform targeting miRNAs. 10 miRNA were upregulated in IPostC vs. I/R while only 5 miRNA was upregulated, but the downregulated miR-29a and miR-29b showed the highest changes.
We plan to publish these results in 2020.

4.6 Effect of rofecoxib treatment on cardiac gene expression profile
Quality control parameters of the reads were within the acceptable range except for the high abundance of mitochondrial tRNAs appearing at the level of overrepresented sequences, read length distribution and GC-content. This deviation however could be likely explained by the high number of mithocondira in the myocardium.
We plan to publish these results in 2020.

4.7 Saliva, plasma miRNA profile analysis
Comparison of the quality control parameters of our saliva and plasma samples to those datasets that were available in the public repositories showed too low miRNA content and high level of noise. Based on these results further improvements are necessary during the RNA-isolation step to achieve better read length distributions and higher miRNA abundances for the purpose of miRNA differential expression analysis.
We plan to publish these results in 2021.

4.8 Relative Entropy Optimization based network visualization software
The software is based on the theoretical basis formulated by István Kovács et al. [13]. The software was implemented previously as a plugin for the well-known Cytoscape network visualization framework. EntOptLayout makes it possible to create network layouts that enable the the researchers to separate functionally important modules of networks even by visual inspection. By calculating the so called normalized relative entropy it also provides an objective approach to measure the quality of the network layout. Several features were also added to improve usability of the software. After implementing these features the software was validated within this project on several networks to prove the effectiveness of the approach. The results were summarized in a recently published paper [2]. MiRNA-target networks arising in studies carried out within this project are all visualized by EntOptLayout, which further proves the usability of this software.
We published these results in an international peer-reviewed journal[2].

5. Summary and conclusion
All aims set in our grant proposal have been achieved by our scientific program. These studies were performed in international collaboration with University of Giessen and Comenius University in Bratislava.
We also recruited 4 PhD and 3 TDK students who were involved in the project. The grant has contributed to 3 accepted and 1 submitted publications in international peer-reviewed journals and 4 publications in preparation. The number of publications will be further increase since we plan to submit manuscripts from the projects. Publishing these results can serve a basis for further projects and scientific grants.

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