

# **Changes in expression of circulating microRNAs after hormone administration and their potential biological relevance**

Ph.D. Theses  
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Budapest  
2015

## I. INTRODUCTION

MicroRNAs (miRNA, miR) are short, non-protein coding, RNA molecules involved in the posttranscriptional regulation of gene expression as parts of the epigenetic machinery. MicroRNAs undergo a complex maturation process giving rise to 17-25 nucleotide long single-stranded mature molecules. Targeting the 3' non-coding region of messenger RNAs (mRNA) and repressing target gene expression in two main manners, mRNA degradation and translation inhibition, they are regarded as endogenous mediators of RNA interference (RNAi).

MicroRNA coding genes make up 1-3 % of known genes, nevertheless this number shows a continuous growth.

Influencing around 30-60% of the protein coding genes, microRNAs play an important role in numerous physiological processes, such as regulation of differentiation, migration, proliferation and apoptosis. They regulate the ontogenesis, the intermediary metabolism, the immune system and homeostasis, as well.

Alterations of tissue microRNA profiles have been described in a wide array of diseases. Their role in carcinogenesis is one of the most extensive fields of microRNA research.

MicroRNAs are implicated in the regulation of almost all phases of carcinogenesis (cell growth, differentiation, proliferation, invasion, apoptosis, angiogenesis, metastasis formation). MicroRNAs can be classified following the classical oncogene–tumor suppressor dichotomy: overexpressed microRNAs in tumors can be regarded as oncogenes, whereas underexpressed microRNAs are tumor suppressors. Besides their role in tumor pathogenesis, their relevance in the diagnosis of various tumors as potential biomarkers is obvious, as there is a significant difference in microRNA profile of benign and malignant tumors.

Recent data appear to add a further layer of complexity to the biological relevance of microRNAs, as secreted microRNAs have been found in body fluids (blood serum or plasma, urine, semen, saliva, etc.) The investigation of the extracellular microRNAs, their relevance as potential biomarkers has been subjected to intensive research efforts, recently.

Besides their role in tumor pathogenesis the question can be raised regarding the potential physiological role of circulating microRNAs in healthy individuals.

In my work, I investigated the potential diagnostic-biological role of circulating microRNAs. In the first part of my theses, I studied the microRNA pool of healthy individuals

and analyzed their potential activity as parts of a tumor surveillance mechanism, and in the second part of my work I investigated the question, whether the routinely used hormone tests affecting the hypothalamo-pituitary-adrenal axis may influence the expression of circulating microRNAs.

## II. AIMS OF OUR STUDIES

1. By analyzing the microRNA profiles of healthy individuals using bioinformatical approach and literature data, I investigated whether the circulating tumor suppressor microRNAs might have a „tumor surveillance” activity.
2. To study the potential changes in expression of selected circulating microRNAs by widely used hormone tests affecting the hypothalamus-pituitary-adrenal axis (Dexamethason suppression, ACTH stimulation). This study included promising circulating microRNA markers of adrenocortical cancer, as their potential changes to hormone treatments might limit their applicability.

### **III. METHODS**

#### **Bioinformatical analysis of circulating microRNAs in healthy individuals**

We have performed an *in silico* analysis of the most abundant microRNAs in blood samples of healthy individuals by downloading datasets from Gene Expression Omnibus ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)). Data of 61 samples from 5 studies have been retrieved. The microRNAs were rank ordered relative to the 20<sup>th</sup> most abundant microRNA. We have also studied literature data down-loaded from the PubMed database ([www.pubmed.org](http://www.pubmed.org)).

#### **Patients and hormone examinations**

In the 2nd Department of Internal Medicine Semmelweis University 10 patients were tested for suspected hypercortisolism by low-dose overnight (1mg) dexamethasone suppression test suffering from obesity, hirsutism, hypertension, and adrenal incidentaloma. Another 10 patients have been examined by 250  $\mu$ g tetracosactide (Cosyntropin, Sandoz Inc.) for suspected Addison's disease or late onset congenital adrenal hyperplasia (deficiency of 21-hydroxylase)

suffering from weakness, secondary oligomenorrhea, infertility, or hirsutism. All tested individuals have been eventually found to be free from any functional disturbance of the hypothalamic-pituitary-adrenal axis.

### **RNA isolation from plasma samples**

Total RNA was isolated with Qiagen miRNeasy Mini Kit (Qiagen GmbH).

### **Real-Time Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) from Plasma Samples.**

RT-qPCR was performed by TaqMan Fast Universal PCR Master Mix (2x) (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol. The samples were amplified on a 96-well plate using a 7500 Fast Real-Time PCR System, according to the manufacturer's protocol. Samples were run in triplicate.

The following probes have been used: *hsa-miR-27a* (000408), *hsa-miR-200b* (002251), ***hsa-miR-214*** (002306), ***hsa-miR-483-5p*** (002338), ***hsa-miR-503*** (001048), and *celmiR-39* (000200) as reference gene. The highlighted circulating

microRNAs have been suggested as markers of adrenocortical cancer.



### **Cell line and *in vitro* treatments, molecular studies**

The NCI-H295R adrenocortical carcinoma cell line was purchased from the American Type Culture Collection and maintained in the recommended media.

For treatments, hormone-free fetal bovine serum (FBS) was used. Dexamethasone treatments were repeated four times. Total RNA was extracted using miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) both from cells and culture medium according to the manufacturer's protocol. RTqPCR reactions were performed by Taqman miRNA Assays (Applied Biosystems) using specific primer/probe combinations: *hsa-miR-27a* (000408) and *cel-miR-39* (000200) as reference gene.

### **Statistical methods**

To identify microRNAs showing significant expression changes, Student's *t*-test or Mann-Whitney *U* test was used depending on the results of Shapiro-Wilks normality test. Statistical analysis of RT-qPCR data was done by Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA) software.

## IV. RESULTS

### **Analysis of microRNA profile in healthy individuals**

We have performed the *in silico* analysis of the most abundant microRNAs in plasma samples of healthy individuals. We ranked the microRNAs according to their expression value, and we expressed the top 20 microRNAs relative to the 20. most abundant one. Besides we studied scientific datas as well.

Using an arbitrary boundary, i.e., over tenfold overrepresented relative to the 20th most common microRNA in the ranking we analysed the top 20 microRNAs. The *hsa-miR-451* (106-fold higher expression than that of the 20th microRNA in the ranking), *hsa-miR-223* (11 to 338-fold), *hsa-miR-16* (11 to 20-fold) and *hsa-let-7f* (16-fold) can be highlighted. Two further microRNAs: *hsa-miR-486-5p* was identified in three independent studies (28.8-fold expression) and *hsa-miR-92*, which showed a 200 fold expression in one study.

Analysing the oncogene-tumor suppressor activity of these microRNAs we have established a hypothesis regarding the potential tumor surveillance activity of these circulating microRNAs.

## **Changes in expression of circulating microRNAs following administration of Dexamethasone and Adrenocorticotropin**

From the five microRNAs selected, only one circulating microRNA, *hsa-miR-27a*, turned out to be significantly modulated by dexamethasone and tetracosactide treatment in our study. Most interestingly, dexamethasone and tetracosactide treatments resulted in opposite changes of *hsa-miR-27a* expression as dexamethasone up-regulated its plasma level, whereas tetracosactide suppressed its expression.

To confirm the dexamethasone responsiveness of *hsa-miR-27a*, we have performed *in vitro* experiments on the adrenocortical NCI-H295R cell line. We have observed that dexamethasone treatment increased the level of secreted *hsa-miR-27a in vitro*, as well.

## V. DISCUSSION

In the first part of my study, I have performed an *in silico* investigation of circulating microRNAs in healthy individuals, and analyzed literature data, as well. Based on my results, the predominantly tumor suppressive microRNAs appear to be overrepresented in the circulation. The possibility might be raised that the circulating tumor suppressor microRNAs might enter transforming cells and thereby inhibit their proliferation, induce cell cycle arrest or apoptosis. I hypothesized that certain sets of circulating microRNAs might function as a tumor surveillance mechanism exerting continuous inhibition on tumor formation complementing the well-described immune tumor surveillance. The tumor surveillance mediated by circulating microRNAs might be active in the early phase of tumor formation. Several counter arguments might be raised against this hypothesis including the dual functions of microRNAs depending on the cellular context, the very low concentration of circulating microRNAs, and the problems associated with their cellular entry. However, I consider that this hypothesis might open a novel aspect for the relevance of circulating microRNAs in healthy individuals. In a further hypothesis, the tissue specific effect of microRNAs has been raised as a putative defense mechanism that could protect cells from the tumor promoting microRNA sets secreted by tumors.

In the second part of my work I studied selected circulating microRNAs based on literature data that either are affected by hormone treatments in animal models or might be potential biomarkers in the diagnostics of adrenal malignancies. As hormone stimulation tests are routinely used in these patients the question was raised if the use of these diagnostic tests might change the expression of these microRNAs.

To the best of our knowledge prior to our study the relationship between the hormones of the hypothalamus-hypohysis-adrenal axis and the circulating microRNAs has not been studied in humans *in vivo*, yet.

Only the expression of *hsa-miR-27a* has been changed by the treatments, its expression was induced by dexamethasone, whereas, tetracosactide inhibited its expression. *In vitro* on an adrenocortical cancer cell line, dexamethasone has induced the expression of *hsa-miR-27a* in culture supernatants, as well. Therefore, the changes of *hsa-miR-27a* levels *in vivo* could be associated with the adrenal cortex. The expression of the promising circulating microRNA markers of adrenocortical cancer, most notably *hsa-miR-483-5p* was not affected by the treatments that underlines its utility in clinical diagnosis.

## VI. CONCLUSIONS

1. Based on the relative abundance of predominantly tumor suppressor circulating microRNAs in healthy individuals, we hypothesized that these microRNAs by conveying epigenetic information to transforming cells might inhibit malignant transformation, and in this way they may participate in the organism's tumor surveillance.
2. The tissue specific effect of microRNAs might represent a putative defense mechanism against tumor formation, as well.
3. We proved the expression's changes of circulating *hsa-miR-27a* after administration of hormones of the hypothalamo-pituitary-adrenal axis (Adrenocorticotropin and Dexamethasone) *in vivo* in humans.
4. Dexamethasone induced secreted *hsa-miR-27a* *in vitro*, as well.

5. The levels of the most promising biomarker at present for adrenal malignancies, the circulating *hsa-miR-483-5p* was not affected by these hormone treatments which confirms its applicability as a potential biomarker in these patients.

## ARTICLES RELATED TO THE PHD THESES

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