

Analysis of the effects of mitotane on gene expression  
and the pathways affected by microRNAs in  
adrenocortical carcinoma

Ph.D. Thesis

**Adrienn Zsippai**

Semmelweis University  
Doctoral School of Clinical Medicine



Programme Chairman: Dr. Károly Rác D.Sc.

Tutor: Dr. Péter Igaz D.Sc.

Opponents: Dr. Zoltán Pócs Ph.D.  
Dr. Erika Hubina Ph.D.

Head of Examination Committee: Dr. Katalin Darvas Ph.D.  
Members of Examination Committee: Dr. Sarolta Gundy Ph.D.  
Dr. Bálint Nagy Ph.D.

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## I. INTRODUCTION

Sporadic adrenal tumours are common, based on pathological data they occur in 7-9% of the population. Their frequency increases with aging. The majority of sporadic tumours originate from the adrenal cortex. Most of these tumours are benign, hormonally inactive adenomas. Hormone-producing adrenal tumours are rare; the most important of these are aldosterone and cortisol producing adenomas. Aldosterone-producing adenomas causes hypertension, hypokalemia and metabolic alkalosis (Conn's syndrome), while overproduction of cortisol leads to central obesity, phenotypic characteristic deviations, hypertension, osteoporosis and diabetes (Cushing's syndrome).

Adrenal cortical carcinoma (ACC) is a rare disease compared to the number of benign adrenal tumours. In advanced stages its prognosis is fairly poor. Intensive research efforts are in progress for identifying novel and efficient treatments, but no significant breakthrough has been reported in this area yet. Mitotane is the only adrenal-specific drug available for ACC treatment at present.

Several issues of the pathogenesis, diagnosis and treatment of adrenal cortical carcinoma are still unclear. Better understanding of the pathogenesis may help tumour classification, diagnosis and effective treatment. High-throughput bioinformatics studies led to significant progress in identifying the pathogenesis of numerous tumours, such as in the case of adrenal tumours. Our team worked in several studies in this area. Besides learning about the pathogenesis, gene expression microarray studies are also useful in examining possible treatment options. We were the first to examine the effects of mitotane on gene expression. Mitotane is used in the treatment of adrenocortical carcinoma, though its mechanism of action is still only partially known.

Low molecular weight non-coding RNA molecules, microRNAs are involved in the post-transcriptional regulation of gene expression, and they represent a new chapter in the molecular pathogenesis of tumours. Our research team was one of the firsts to report their differing expression experienced in adrenal tumours. There are significant differences between the results of studies examining the gene expression of microRNAs. A comprehensive bioinformatics study of disease processes influenced by microRNAs has not been made yet. In my thesis I attempted to provide a comprehensive analysis that describes the biological significance of microRNA profiles reported in different adrenal tumour studies.

## II. AIMS OF OUR STUDIES

1. Examining the complete *in vitro* effects of mitotane genome gene expression in an adrenocortical carcinoma cell line.
2. In particular, we examined the question of whether gene expression effects play a role in mitotane's steroid hormone biosynthesis inhibitory effect.
3. By summarizing the results of studies published so far describing the microRNA expression pattern; we prepared a bioinformatic analysis of disease processes that are influenced by the significantly differentially expressed microRNAs.

### III. METHODS

#### Cell cultures and treatments

Human adrenocortical carcinoma NCI-H295R cells were cultured as a monolayer in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (DMEM:F-12) medium supplemented with insulin, transferrin, selenium, linoleic acid, bovine serum albumin, Penicillin/Streptomycin, Nu-Serum, L-Glutamine and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Sigma-Aldrich Chemical Co.). Cells were cultured in a humidified environment at 37°C and subcultured every 7 days. Cells were treated with mitotane for 24, 48, 72 and 96 hours at  $10^{-4}$ M,  $10^{-5}$ M,  $5 \times 10^{-6}$ M and  $10^{-6}$  M concentrations. The control group was treated with the same volume and concentration of ethanol.

#### Cell viability analysis

For defining cell viability we used MTT test and flow cytometry assay.

In the MTT test, NCI-H295R cells were plated at a density of  $1 \times 10^4$  cells per well, in complete culture medium. After 48 hours of incubation, cultures were incubated again in  $10^{-4}$  M,  $10^{-5}$  M,  $5 \times 10^{-6}$  M,  $10^{-6}$  M mitotane medium for 24, 48, 72 and 96 hours. Using 20  $\mu$ l MTT (Sigma-Aldrich Chemical Co.) and further 24 hours of incubation optical density was measured with Labsystem Multiscan Multisoft microplate reader (Labsystem).

For the flow cytometry assay, NCI-H295R cells were plated at a density of  $1.5 \times 10^5$  cells per well in complete medium. After 48h incubation, cells were treated with  $10^{-4}$  M,  $10^{-5}$  M,  $5 \times 10^{-6}$  M and  $10^{-6}$  M concentration mitotane for 24h, 48h, 72h and 96h. Then, cells were trypsinized with 0.5 g/L trypsin (Sigma-Aldrich Chemical Co.) and samples were measured with a FACSCalibur (BD Biosciences) cytometer. Data were analyzed using CellQuest ProTM software (BD Biosciences).

Cells treated with the same amount of ethanol were used as control in both of these measurements.

#### Determining steroid hormone levels

After 48h incubation NCI-H295R cells were treated with  $10^{-6}$  M mitotane for 24, 48, 72 and 96 hours; cortisol and androstenedione level measurements were performed using the cells' supernatants. Cortisol level was measured on an Elecsys Immunoanalyser System (Roche Diagnostics Ltd.) by the Roche Cobas Cortisol electrochemiluminescence immunoassay

(Roche Diagnostics GmbH). Androstenedione level was measured using Androstenedione Radioimmunoassay Kit (DiaSorin SPA).

### **RNA isolation**

RNA was isolated from  $2 \times 10^6$  mitotane treated ( $5 \times 10^{-6}$  M) and ethanol treated NCI-H295R cells with Qiagen miRNeasy Mini Kit (Qiagen GmbH), whereas DNase digestion was carried out with the RNase-Free DNase Set (Qiagen GmbH).

RNA concentration was determined by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.) and RNA integrity was examined by an Agilent 2100 Bioanalyzer System (Agilent Technologies Inc.).

### **mRNA expression profiling**

16 independent NCI-H295R samples (4 control samples at 48h, 4 control samples at 72h, 4 samples of mitotane-treated at 48h and 4 samples of mitotane-treated at 72h) have been subjected to mRNA expression profiling on 4x44K Agilent Whole Human Genome Microarray slides (Agilent Technologies Inc.).

### **QRT-PCR examinations**

Validation of genes showing significant expression differences was performed after increasing the number of cases in each group ( $n=6$ ), by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) method. 7 significant gene ( $p < 0.05$ ) expression abnormalities were selected for validation and increasing number of cases. Based on the analysis of the least fold change difference and coefficient of variation, ZNF625 (Applied Biosystems) has been identified as the most appropriate housekeeping gene.

### **Statistical analysis**

Statistical analysis of microarray data was performed by GeneSpring 10.1 (Agilent Technologies Inc.) software. Identification of genes differentially expressed in microarray analysis was carried out by two-ways ANOVA, Student t-test followed by Tukey's Honestly Significant Difference post-hoc test and Benjamini-Hochberg correction. Statistical analysis of viability, steroid hormone levels and qRT-PCR data was performed by Microsoft Office Excel 2010 (Microsoft Corp.), and Statistica 8.0 (StatSoft Inc.) softwares. qRT-PCR data were subjected to two-ways ANOVA and Tukey's post-hoc test.

## **Bioinformatic analysis of pathogenetic pathways influenced by microRNAs**

### **Datasets**

We collected the miRNA expression data of ACC, ACA and normal adult adrenals from five studies. Thus, a total of 631 miRNAs were identified, of which 305 miRNAs were overexpressed and 326 were underexpressed miRNAs. Raw miRNA expression data were grouped as the following: overexpressed ACA miRNAs vs. overexpressed ACC miRNAs; underexpressed ACA miRNAs vs. underexpressed ACC miRNAs; overexpressed Normal miRNAs vs. overexpressed ACC miRNAs; underexpressed Normal miRNAs vs. underexpressed ACC miRNAs. In the further trials we used miRNAs those were included in at least two studies.

miRNA expression data on ACC, ACA and normal adrenals have been collected from three studies. All of these studies have been identified by literature search (PubMed, [www.ncbi.nlm.nih.gov/pubmed](http://www.ncbi.nlm.nih.gov/pubmed)).

### **Tissue specific miRNA target prediction**

Potential mRNA targets of miRNAs those proved to be significant in at least two studies were identified with using three target prediction computer algorithms (TargetScan 5.2, PicTar and Microcosm Targets). The results were summarized by an own software written in Java programme language.

To achieve tissue specificity, results of mRNA expression studies have been used alongside miRNA data. After filtering we used Gene Set Enrichment Analysis (GSEA, [www.broad.mit.edu](http://www.broad.mit.edu)) with Leading Edge Analysis (LEA) approach.

### **Pathway Analysis**

Pathway analysis was performed on GSEA-filtered tissue specific miRNA targets using Ingenuity Pathway Analysis (IPA) 7.1 Software (Ingenuity Systems).

## IV. RESULTS

### Cell viability analysis

Based on the results of cell viability examinations we found that treatment with  $10^{-5}$ ,  $5 \times 10^{-6}$  and  $10^{-6}$  M of mitotane had no significant effect on cell viability after 24, 48, 72 and 96 h incubation time. However, mitotane at  $10^{-4}$  M concentration dramatically decreased cell viability both in MTT and in flow cytometry examinations.

### Measuring steroid hormone levels

By measuring cortisol and androstenedione levels we found that in NCI-H295R adrenal carcinoma cell lines  $5 \times 10^{-6}$  M mitotane concentration is the optimal treatment concentration that effectively inhibits hormone synthesis without having any effects on cell viability.

### Microarray Analysis

Based on the results of hormone measurements showing over 50% inhibition of both hormones tested at 48 h, treatment periods of 48 and 72 h were chosen for the further analysis of gene expression. In our analysis  $5 \times 10^{-6}$  M mitotane concentration was chosen.

With more than two-fold expression change, we identified a total of 117 significantly expressed genes in the 48-hour and 72-hour mitotane treated groups compared to the corresponding control groups. Based on these results, we detected 63 significantly differentially expressed genes in the 48-hour treated samples and 111 genes in the 72-hour samples. Without considering time factor, we identified 60 significantly differentially expressed genes.

After identifying the genes with significant differences based on the microarray, we validated 7 genes with qRT-PCR method. As during our research we wished to examine the potential effects of mitotane on steroid hormone biosynthesis, for validation we chose three genes participating in steroid hormone biosynthesis showing the greatest change (the mostly underexpressed ones): *HSD3B1*, *HSD3B2* and *CYP21A2*. In addition we also identified the four genes with the highest overexpression change values: *GDF-15*, *ALDH1L2*, *TRIB3* and *SERPINE2*.

### **Validating the expression of selected genes with quantitative real-time PCR method**

In our study we identified a strong expression increase of *GDF-15*, *ALDH1L2*, *TRIB3* and *SERPINE2* genes in the mitotane-treated group compared to control group in both 48-hour and 72-hour treatment times. *GDF-15* showed the highest value of increased expression in both 48- and 72-hour groups. Overexpression of *TRIB3* gene was significant in mitotane treated samples compared to control samples in both 48- and 72-hour groups. Underexpression of *HSD3B1* and *HSD3B2* genes was significant in mitotane treated samples compared to control samples in both 48- and 72-hour groups. Underexpression of *CYP21A2* was significantly different in the 72-hour groups.

### **Bioinformatic analysis of pathogenetic pathways influenced by microRNAs**

#### **Tissue specific target prediction**

After statistical analysis, a total of 49,817 mRNA molecules have been predicted to be targets of the 39 significantly different (under- or overexpressed) miRNAs common in at least two published studies, which means a total of 29,079 different targets. 23,010 mRNAs were predicted by TargetScan, 1771 mRNAs by Pictar and 8913 mRNAs by MicroCosm.

#### **Pathway analysis**

We identified a total of 820 pathways with Ingenuity Pathway analysis those were affected by miRNAs common in at least two different studies. Among these we identified 418 significant pathways based on p-value ( $p < 0.05$ ); 178 of these participated in all studies. Based on our previous meta-analysis we chose 12 of pathways involved in retinoic acid signaling and cell cycle regulation for further analysis.

#### **Retinoic acid signaling pathways**

In the ACC-Normal comparison we identified peroxisome proliferator-activated receptor alpha/retinoic X receptor alpha (*PPAR $\alpha$ /RXR $\alpha$* ) and peroxisome proliferator-activated receptor (*PPAR*) signaling pathways, whereas in the ACA-ACC comparison we identified lipopolysaccharide/interleukin-1 (*LPS/IL-1*) mediated inhibition of retinoic X receptor (*RXR*) function, *PPAR $\alpha$ /RXR $\alpha$*  activation and retinoic acid receptor (*RAR*) activation pathways.

In the Normal-ACC comparison the mRNA expression changes of retinoic acid signaling pathways may be related to the following overexpressed (*miR-127-3p*, *miR-184*, *miR-210*, *miR-424*, *miR-432*, *miR-483-5p*, *miR-487b*, *miR-503*) and underexpressed (*miR-214*, *miR-511*, *miR-375*) miRNAs.

In the ACA-ACC comparison the mRNA expression changes of retinoic acid signaling pathways may be related to the following overexpressed (*miR-106b*, *miR-127-3p*, *miR-130b*, *miR-135a*, *miR-136*, *miR-148b*, *miR-184*, *miR-210*, *miR-376c*, *miR-410*, *miR-424*, *miR-432*, *miR-483-5p*, *miR-487b*, *miR-503*, *miR-506*, *miR-542-3p*, *miR-542-5p*, *miR-642*, *miR-450a*) and underexpressed (*miR-let-7b*, *miR-101*, *miR-125b*, *miR-195*, *miR-214*, *miR-497*, *miR-557*, *miR-600*, *miR-617*, *miR-199a-3p*, *miR-199a-5p*, *miR-202*, *miR-335*, *miR-511*, *miR-572*, *miR-647*, *miR-708*, *miR-99a*) miRNAs.

### **Cell cycle regulatory pathways**

In case of Normal-ACC comparison we identified the following cell cycle regulatory pathways: the aryl hydrocarbon receptor, the DNA damage-inducible 45 (GADD45) signaling pathway and the integrin signaling pathway, while in the ACA-ACC comparison we identified the integrin signaling pathway, the G2/M damage checkpoint regulation, the cell cycles control of chromosomal replication and the cyclins and cell cycle regulation pathways.

In the Normal-ACC comparison the mRNA expression changes of aryl hydrocarbon receptors and GADD45 signaling pathways may be in connection with the following overexpressed (*miR-127-3p*, *miR-424*, *miR-432*, *miR-483-5p*, *miR-503*, *miR-184*, *miR-210*, *miR-487b*) and underexpressed (*miR-214*, *miR-375*, *miR-511*) miRNAs. The mRNA expression changes of the integrin signaling pathway may be related to the following overexpressed (*miR-127-3p*, *miR-184*, *miR-423*, *miR-424*, *miR-483-5p*, *miR-487b*, *miR-503*, *miR-210*, *miR-432*) and underexpressed (*miR-214*, *miR-51*, *miR-375*) miRNAs.

In the ACA-ACC comparison mRNA expression changes of integrin signaling pathway may be in contact with the following overexpressed (*miR-106b*, *miR-127-3p*, *miR-135a*, *miR-136*, *miR-148b*, *miR-376c*, *miR-424*, *miR-432*, *miR-503*, *miR-506*, *miR-542-3p*, *miR-542-5p*, *miR-642*, *miR-130b*, *miR-210*, *miR-410*, *miR-483-5p*, *miR-487b*, *miR-450a*) and underexpressed (*miR-let-7b*, *miR-101*, *miR-125b*, *miR-195*, *miR-199a-3p*, *miR-199a-5p*, *miR-202*, *miR-214*, *miR-335*, *miR-511*, *miR-557*, *miR-600*, *miR-617*, *miR-647*, *miR-708*, *miR-99a*, *miR-497*, *miR-572*) miRNAs. The mRNA expression changes of the G2/M damage checkpoint regulation, the cell cycles control of chromosomal replication, the cyclins and cell

cycle regulation may be related to the following overexpressed (*miR-135a*, *miR-487b*, *miR-410*, *miR-503*, *miR-106b*, *miR-127-3p*, *miR-148b*, *miR-184*, *miR-424*, *miR-483-5p*, *miR-506*, *miR-542-3p*, *miR-542-5p*, *miR-642*) and underexpressed (*miR-let-7b*, *miR-101*, *miR-125b*, *miR-195*, *miR-199a-3p*, *miR-199a-5p*, *miR-202*, *miR-214*, *miR-335*, *miR-497*, *miR-511*, *miR-557*, *miR-572*, *miR-600*, *miR-617*, *miR-647*, *miR-708*, *miR-99a*) miRNAs.

## V. DISCUSSION AND CONCLUSIONS

Our group was the first to investigate the whole genome gene expression effects of mitotane that is commonly used in adrenocortical carcinoma, though its mechanism of action is still unclear.

My main results are the following:

- We defined that in NCI-H295R adrenal carcinoma cell lines  $5 \times 10^{-6}$  M mitotane concentration is the optimal therapeutic concentration that inhibits hormone synthesis effectively without affecting cell viability.
- We applied this treatment concentration for 48-72 hours of treatment time, and our microarray analysis identified a total of 117 significantly differentially expressed genes.
- We validated the highest over- and underexpressed genes of these (n=7) with qRT-PCR method.
- Three of these are significantly underexpressed genes playing an essential role in hormone biosynthesis (*HSD3B1*, *HSD3B2* and *CYP21A2*). Based on this, beside the well-known direct enzyme inhibitory effects, the inhibition of gene expression of steroid hormone biosynthetic enzymes also play a role in the steroid hormone biosynthesis inhibiting effect of mitotane.
- At present it is difficult to relate the mostly overexpressed genes (*GDF-15*, *ALDH1L2*, *TRIB3* and *SERPINE2*) to the pharmacological effect of mitotane, as - according to our latest knowledge - their gene expression changes tend to show tumour growth-promoting effect. Further testing will be required for clarify the importance of these genes in mitotane effect.

In adrenocortical carcinomas, a comprehensive bioinformatics analysis of disease processes influenced by microRNAs has not yet been reported. Therefore, in the second part of my dissertation I attempted to describe the biological significance of microRNAs showing significant changes in adrenocortical tumours, described in at least two studies.

- Based on statistical analysis: we predicted 39 significantly altered miRNAs, 49817 mRNAs and 29079 different targets.
- With using Ingenuity Pathway analysis we identified 418 pathways showing significant changes ( $p < 0.05$ ); based on our earlier investigation, pathways involved in retinoic acid signaling and cell cycle regulation were subjects of further analysis ( $n=12$ ).
- We found that mRNA expression changes of these pathways may be related to microRNAs showing significant changes.
- These pathways are:
 

In the Normal-ACC comparison: the peroxisome proliferator-activated receptor alpha/retinoic X receptor alpha (*PPAR $\alpha$ /RXR $\alpha$* ) pathway, the peroxisome proliferator-activated receptor (*PPAR*) signaling pathway, aryl hydrocarbon receptor pathway, the DNA damage-inducible 45 (*GADD45*) signaling pathway and the integrin signaling pathway.

In the ACA-ACC comparison: lipopolysaccharide/interleukin-1 (*LPS/IL-1*) mediated Inhibition of retinoic X receptor (*RXR*) function, *PPAR $\alpha$  /RXR $\alpha$*  activation pathway, the retinoic acid receptor (*RAR*) activation pathway, the integrin signaling pathway, the G2/M damage checkpoint regulation, cell cycles control of chromosomal replication, cyclins and cell cycle regulation pathway.

However, we must emphasize that we identified the pathogenic pathways with bioinformatics method, and they should be considered only as *in silico* predictions, therefore their further experimental *in vitro* validation is essential.

## ARTICLES

### ARTICLES RELATED TO THE PHD THESIS

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