Genetic and epigenetic biomarkers in pituitary adenomas

Ph.D. theses

Kinga Fülöpné Németh

Semmelweis University
Clinical Medicine Doctoral School

Advisor: Dr. Henriett Butz, Ph.D.

Thesis reviewers:
Dr. Anna Sebestyén, Ph.D.
Dr. Erika Hubina Ph.D.

Final Exam Committee:
Head: Dr. András Kiss, D.Sc.
Members: Dr. Zsuzsanna Putz, Ph.D.
Dr. Krisztina Takács, Ph.D.

Budapest
2019

I. Introduction

Pituitary adenomas are common neoplasms accounting for 10-15% of all intracranial tumors. The estimated prevalence for clinically relevant pituitary adenomas is approximately 1:1000 in the general population. Due to the altered hormonal secretion and mass effect they can cause significant morbidity. Approximately 70% of them are functioning, that can cause clinical manifestation through their hormonal activity (e.g. acromegaly, Cushing's disease). The other 30% are non-functioning (hormonally inactive) adenomas, where even there is positive hormone staining in immunohistochemistry, clinical symptoms are absent. Besides, some show positivity to several hormones (plurihormonal adenomas), or completely lack of anterior pituitary hormone positivity (hormone-immunonegative).

95% of pituitary adenomas are sporadic and the molecular mechanism of their pathogenesis is largely unknown. The better understanding of these mechanisms would be essential to identify new biomarkers and therapeutical applications.

Eukaryotic tissues usually contain approximately 100 mitochondria per cell, all of which encompass several copies of 16569 nucleotide-long circular mitochondrial DNA (mtDNA). Mitochondria provide most of the energy of the cells through oxidative phosphorylation, and have a role in apoptosis, thus disruption of mtDNA can lead to errors in cell functions. The first tumor-associated variants have been identified in 1998 in colorectal carcinoma. Since then, mitochondrial variants have been identified in a variety of cancer tissues and analysed their potential role in tumorigenesis.

MiRNAs are short single-stranded RNA molecules that have an important role in the fundamental biological functions. In the last years, due to their high stability in blood, extracellular miRNAs as non-invasive biomarkers have become an important factor in biomedical research. Increasing number of studies show association between circulating miRNAs and endocrine tumors, however they have not been analysed in association with pituitary adenomas yet. Because miRNAs and extracellular vesicle associated miRNAs are secreted by almost every cell type their origin in circulation is difficult to prove. Yet there are studies showing tumor-associated miRNAs with a higher level in circulation. Nevertheless, miRNAs, consistently showing higher or lower level in circulation in association with the tumor presence – independently of their origin – can be used as biomarkers.

Among pituitary adenomas, prolactinomas can be often treated by dopamine agonists (e.g. bromocriptine, cabergoline), while the first-line therapy of other types of pituitary adenomas is surgical removal. However, total resection is often difficult, especially in the case of tumors with extended size or presenting invasion into adjacent structures

Since dysregulated apoptosis can be a key factor of tumor formation, inducing apoptosis is an emerging path in developing tumor therapies. Human recombinant TRAIL (TNF-related apoptosis-inducing ligand) treatment is a promising approach, because of its selectivity to tumor cells. However, several cell lines show resistance to TRAIL, which can at least partially due to survivin overexpression.

Survivin is part of the inhibitor of apoptosis protein family (iAPs) and have role both in apoptosis and cell cycle regulation. It is usually hardly or not expressed in adult tissues, but it exhibits overexpression in a variety of cancers. Moreover, it has been associated with resistance to therapy and poor survival. Thus, survivin is a promising therapeutical target in neoplastic diseases.

II. Aims

The objectives of my Ph.D. study were:

1. Characterization of the whole mitochondrial genome in pituitary adenomas by next generation sequencing (NGS) and investigating mitochondrial DNA variants to better understand the molecular mechanisms behind pituitary adenoma pathogenesis.

2. I aimed to identify new biomarkers of pituitary adenomas by analysing circulating miRNAs in plasma samples. My goal was to:

    a. compare miRNAs and isomiRs between pituitary adenoma patients and healthy individuals,

    b. investigate miRNAs in pre-, and postoperative plasma pairs of pituitary adenoma patients.

3. My third goal was to investigate potential therapeutic targets based on gene expression measurements of pituitary adenoma tissues. Meaning:

    a. investigating the expression and function of survivin and TRAIL molecules

    b. *in vitro* analysis of the effect of acetylsalicylic acid on pituitary adenoma cells through survivin inhibition.

    c. I also aimed to confirm my results *in vivo* and creating xenograft model for gonadotroph pituitary adenoma using RC-4 B/C cell line.

III. Materials and Methods

III.1. Patients and samples

A total of 102 pituitary adenoma (PA) and 10 normal pituitary (NP) tissue specimens were analysed, and 149 plasma samples were obtained from 45 patients, and 2 additional plasma samples from healthy individuals.

Patients were recruited in the 2nd Department of Internal Medicine, Semmelweis University, Hungary. Pituitary adenomas were surgically removed by transsphenoidal surgery at the National Institute of Clinical Neurosciences between 2015 and 2017. Normal pituitary samples were obtained by autopsy within 6 hours of death from patients with no evidence of any endocrine disease in a frame of a previous collaboration (2009) (University Clinical Centre, Belgrade, Serbia).

III.2. Isolation of extracellular vesicles and nucleic acids

Extracellular vesicles (EVs) were extracted from 300 ul plasma samples using Total Exosome Isolation Kit (from plasma) (Thermo Fisher Scientific). DNA was isolated from 45 pituitary adenoma tissues using QIAamp Fast DNA Tissue Kit (Qiagen) and from cells using QIAamp DNA Mini Kit (Qiagen). For RNA isolation from cell and tissue samples RNeasy and miRNeasy Mini Kits (Qiagen), from plasma and exosome samples miRNeasy Serum/Plasma Kit (Qiagen) were used. As a spike-in control cel-miR-39-3p exogen miRNA (Invitrogen) was added during isolation. Nucleic acid concentration was measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

III.3. Sequencing

For mitochondrial genome analysis library preparation was performed from genomic DNA using VariantProTM Mitochondrion Panel Library Preparation Kit (VP-MIT-0048, LC Sciences, LLC). For miRNA sequencing small RNA library was prepared using QIAseq^{TM} miRNA Library Kit (Qiagen).

The presence of the desired fragments and the purity of the indexed libraries were analysed on Agilent 2100 Bioanalyzer (Agilent Technologies) using High-Sensitivity DNA Analysis Kit (Agilent Technologies). The concentrations of the libraries were measured using Qubit Fluorometer (Thermo Fisher Scientific). Equimolar amounts of the indexed libraries were pooled to obtain a 4 nM library mixture. After denaturing by sodium-hydroxide and further dilution, the final 10 pM of library mixture was loaded into Illumina cartridge.

For mitochondrial DNA sequencing Illumina MiSeq Reagent v2 kit (500 cycles), for miRNA sequencing Illumina MiSeq Reagent v3 kit (150 cycles) were used. Sequencing reactions were performed on the Illumina MiSeq instrument (Illumina).

The validation of the single-nucleotide variants (SNVs) was performed by Sanger sequencing. We sequenced the same 45 tissue samples used in NGS. To avoid co-amplification of nuclear DNA, a well-established method was used for the exclusive amplification of mitochondrial fragments. The specific mitochondrial DNA fragments were PCR-amplified, and Sanger sequencing run was performed on Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific) using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher Scientific).

III.4. Gene and miRNA expression measurements

Expression changes of apoptosis genes were measured in 29 NFPA, 19 GHPA and 10 NP tissue specimens with TaqMan Low Density Array (TLDA) (Applied Biosystems), and miRNA expression was measured in 6 GHPA and 5 NP tissues with TaqMan Low Density Array (TLDA) Human MicroRNA Panel v.2 (Applied Biosystems).

For individual gene and miRNA expression measurements RNA samples were reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific).

Survivin gene and miRNA expressions were measured using predesigned TaqMan gene expression assays (rno-BIRC5: Rn00574012_m1, hsa-BIRC5: Hs04194392_s1, hsa-ACTB: Hs99999903_m1, 18S: Hs99999901_s1, cel-miR-39-3p: 478293_mir, hsa-143-3p: 477912_mir, hsa-6867-5p: 480488_mir,150 hsa-6514-3p: 480214_mir, hsa-150-5p: 477918_mir, hsa-126-5p: 477888_mir, hsa-26b-5p: 478418_mir, hsa-151 148b-3p: 477824_mir) and TaqMan Universal Master Mix (Thermo Fisher Scientific).

Ciklin A2 (CCNA2) and CDK2 gene expressions were measured using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and custom designed primers (rno-CCNA2 forward: GGATGGTAGTTTTGAATCACCCC, reverse: GGATGGCCCGCATACTGTTA, rno-CDK2 forward: GCTTATCAACGCAGAGGGGT and reverse: GGGTCACCATTTCGGCAAAG, rno-ACTB forward: AGATCAAGATCATTGCTCCTCCT and reverse: ACGCAGCTCAGTAACAGTCC).

All measurements were performed in triplicates on 384-well plates using a Quant Studio 7 Flex Real-Time PCR Systems (Thermo Fisher Scientific). The expression level was calculated by the ddCt method.

III.5. Western blot analysis

Protein samples were mixed with 5x sample buffer (Thermo Fisher Scientific), denatured at 99°C for 5 minutes and separated on 15% SDS polyacrylamide gel electrophoresis, transferred to a PVDF membrane and incubated overnight with primary antibodies: survivin (1:500; #2808), CDK2 (1:1000; #2546), p-CDK2 (1:1000; #2561) (Cell Signaling), cyclin A2 (1:1000; #MA1-154) (Thermo Fisher Scientific). For loading control membranes were stripped and re-probed using mouse anti-β-actin (1:2000, Cell Signalling Technology). Anti-mouse (1:2000, #P044701, Agilent) and anti-rabbit (1:2000, #P044801, Agilent) HRP-conjugated IgGs were used as secondary antibodies and detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) and Kodak Image Station 4000MM. Band intensities were quantified using Image J software (Bethesda).

III.6. Immunohistochemistry

FFPE tissue sections of 16 NFPAs, 9 GH-producing pituitary adenomas, 5 normal adenohypophysis and a lymph node as positive control were selected for immunohistochemical analysis. 4μm-thick sections were immunostained following standard procedure. Anti-survivin antibody (71G4B7, #2808, Cell Signaling Technology) was used at 1:4000 dilution. Goat anti-rabbit immunoglobulin (#P0448, Agilent, 1:200) was applied as secondary antibody. Slides were developed with DAB (Novocastra Laboratories) and counterstained with haematoxylin. The stained slides were digitally scanned with a high-resolution scanner (Pannoramic Scan, 3DHISTECH Ltd.), and used for virtual microscopic evaluation and quantification with NuclearQuant module of the CaseViewer v2.1 software (3DHISTECH Ltd.). Survivin staining was scored negative or weak, medium or strong positive, and the percentage of nuclei with each staining intensity per case were calculated by the software. Medium and strong staining was used for analysis in comparison of adenoma and normal tissues.

III.7. Cell culture analyses

RC-4 B/C (CRL-1903) and GH3 (CCL-82.1) cells were purchased from American Type Culture Collection (ATCC). Acetylsalicylic acid (ASA) was used in 1-5 mM, YM155 in 0.1-5 uM and TRAIL in 2 ug/ml concentrations. All experiments were repeated 3 times independently.

Cell proliferation was assessed by adding 10% Alamar Blue reagent (Thermo Fisher Scientific) and incubating at 37°C for 1 hour and fluorescent measurement was performed on plate reader (Thermo Fisher Scientific). Cell viability was determined using trypan blue staining.

Caspase-3 activity of cells was determined using a Caspase-3 Colorimetric Protease Assay Kit (Thermo Fisher Scientific) and apoptosis was further evaluated by DNA degradation analysis on agarose gel electrophoresis.

Cell cycle analysis was performed using flow cytometry. Cells were stained with 10 ng/ml propidium-iodide and at least 10000 events were measured from each sample using FACSCalibur flow cytometer (BD Sciences). The cell cycle distribution of cells and the level of apoptosis were determined using ModFit and Cell Quest Pro software.

To generate survivin-overexpressing plasmid, survivin fragment was subcloned into a pcDNA3.1 vector (Thermo Fisher Scientific) using XbaI and HindIII-HF restriction enzymes (New England Biolabs). After heat shock transformation of chemical competent Escherichia coli DH5α, bacteria were plated on ampicillin-containing LB agar and grown at 37°C overnight. Next day colonies were checked and two of the positive clones were separately grown in liquid LB at 37°C overnight. For isolation of recombinant DNA PureLink HiPure Plasmid Midiprep Kit (Thermo Fisher Scientific) was used. Before transfection, the sequence of the construct was confirmed with bidirectional Sanger sequencing using the following primers (5'-3') located on the vector: forward primer: AGAACCCACTGCTTACTGGC, reverse primer: GGCAAACAACAGATGGCTGG.

Plasmid was transfected into RC-4 B/C cells using X-tremeGENE HP DNA Transfection Reagent (Sigma). Survivin overexpression was assessed by RT-qPCR and western blot. Transfection efficiency was about 80% evaluated by GFP-containing plasmid transfection and fluorescent microscope.

For survivin silencing, cells were transfected with 2 different Locked Nucleic Acid small interfering RNAs (siRNAs) against survivin (Silencer Select s133761, s133762, Thermo Fisher Scientific) or a negative control siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific). All siRNAs were used in 10 nM final concentration. Gene knockdown was verified by immunoblot analysis.

III.8. Xenograft experiments

Inbred SCID mice were used for generating GH3 and RC-4 B/C xenograft models. 6-8-week-old male mice were subcutaneously injected with GH3 and RC-4 B/C cells mixed with Matrigel Matrix (Corning) in one flank. As RC-4 B/C xenograft has been never generated before, we performed xenograft injection with gradually increasing cell number ($5x10^6$, 10^7 and

$2x10^7$/injection). We used two mice in every experiment and repeated it three times. As a positive control we also injected additional mice with GH3 cells ($1x10^7$) as positive control.

III.9. Bioinformatical and statistical analyses

The results of next generation sequencing (NGS) were analysed by complex bioinformatical approaches. For mitochondrial genome analysis Revised Cambridge Reference Sequence (rCRS) of the Human Mitochondrial DNA (NC_012920.1 gi:251831106) was used as reference. To identify heteroplasmy 3% was used as a cut-off.

To analyse miRNA sequencing data after adapter trimming reads were aligned to miRNA sequences of miRBase online database (www.mirbase.org). Reads were normalized to total read number in each sample and given as read count per 10000 read (normalized read number). IsomiR analysis were done using isomiR-SEA algorithm.

Statistical analyses and interpretations were done using R statistical program and GraphPad Prism 6 (GraphPad Software Inc.). Hierarchical cluster analysis with Euclidean distance method was used for cluster analysis in Pheatmap R package. For comparison between two groups Student's T-test, paired T-test or Mann-Whitney test was used based on the results of Shapiro-Wilk normality test. We adjusted p-values for multiple comparisons with the Benjamini and Hochberg method (BH). To compare several groups one-way ANOVA followed by Tukey post-hoc test or Kruskal-Wallis test followed by Dunn's test was used depending on the results of Shapiro-Wilk normality test. P-values < 0.05 were considered significant.

IV. Results

IV.1. Analysis of mitochondrial genome in pituitary adenomas

The whole mitochondrial genome was analysed in 12 GHPA and 33 NFPA comprising 22 FSH/LH+ and 11 hormone-immunonegative (HN) tumors.

Sequencing run generated an average 52 399 reads per sample (minimum: 24 019, maximum: 242 870) and 95 ± 1% of the reads were aligned to the mitochondrial genome. Coverage depth was 630 ± 370 (avg ± SE) reads per base.

We detected 496 single-nucleotide variants (SNVs) in all tumors. Of these, 269 variants were protein-coding of which 136 were non-synonymous and 133 were synonymous.

We compared our results to previous data in HmtDB database (http://www.hmtdb.uniba.it) analysing GHPA adenomas and obtained that 82 of the detected 496 variants were common and the rest 414 were novel.

Heteroplasmy occurred in 482 of the detected 496 variants at least in one sample and overall on low level. HN adenomas showed a slightly higher (8.27%) heteroplasmy prevalence compared to FSH/LH+ (6.96%) and GH+ adenomas (6.72%). This remained the same after dissection of protein-coding and non-coding variants. In addition, we did not find difference in Ki-67 proliferation index or tumor size between sample groups harbouring low (< 50%) and high (> 50%) heteroplasmy.

On average, 35 variants were detected per sample. Similar results were obtained in different histological groups (GHPA: 33, FSH/LH+ adenoma: 34, HN adenoma: 40). Although number of variants did not show correlation with Ki-67 index, samples harbouring the highest number of variants had the highest Ki-67 indices independently of histological type (GHPA: 8%, FSH/LH+ adenoma: 7-10%, HN adenoma: 5%).

When analysing distinct histological groups, we identified 143, 58, and 52 unique variants that appeared only in FSH/LH+, HN, or GH-secreting adenomas.

Nonparametric ANOVA identified eight significant variants among different histological groups. The variants: A11251G, T4216C, T16126C, C15452A, T14798C, A188G, and T16093C differentiated GH-producing from HN adenomas. The prevalence of variants: T14798C, G185A, A188G, and T16093C differed between FSH/LH+ and HN adenomas. Four (G185A, A188G, T16093C, and T16126C) were localized in the D-loop region. Among the other four variants, which were located in the coding region, A11251G was synonymous, while T14798C, C15452A and T4216C were non-synonymous variants, and were located in CYB

and ND1 genes. These variants have been already known as rs28357681, rs527236209 and rs1599988.

Individual variant prevalence did not show associations with Ki-67 proliferation index or tumor size. However T16189C variant was found in 40% (6/15) of non-recurrent adenomas compared to recurrent ones, where it was not detectable (0/11) ($p = 0.021$).

We selected two, non-synonymous variants (T14798C, T4216C) and validated them on all the 45 samples and we found 100% concordance between NGS and Sanger sequencing results.

IV.2. Identification of circulating miRNA biomarkers in pituitary adenoma patients

To identify potential circultaing miRNA biomarkers pre-, and postoperative plasma pairs or pituitary adenoma patients were analysed by NGS and validated by RT-qPCR. Plasma samples were grouped by the histological type of the removed adenoma, respectively: GH-producing (GH+) group, FSH/LH+ group, hormone-immunonegative (HN) group. Plasma sample of two healthy volunteers (N) were used as controls.

NGS was performed on 36 paired plasma samples (FSH/LH+: 10 pairs, GH+: 4 pairs, HN: 4 pairs) obtained before and 3 months after transsphenoidal pituitary surgery.

On average, 4001268 reads were generated per sample and 41% aligned to known mature miRNA sequences present in miRBase v21. Interestingly, we found that in adenoma groups the overall plasma miRNA read number was lower compared to those detected in plasma samples from healthy individuals (1.3×10^6 vs. 2.3×10^6; $p=0.0392$). 29 miRNAs were revealed which could differentiate plasma samples obtained from patients with different pituitary adenoma subtypes and normal plasma samples

In contrast to previously used methods NGS allows us to detect miRNA variants (called isomiRs). In our plasma samples 53.8%. of total sequencing reads aligned exactly on miRBase mature miRNA sequences Therefore, almost half of the miRNA (46.2%) molecules are present in isomiR form that emphasizes their importance. 40.6% of the reads were 3p isomiRs, 1.6%, 1.2% were 5p and SNP isomiRs, respectively, while 2.8% of the reads contained multiple variants at the same time.

Altogether we found only 11 miRNAs with different isomiR variant distribution comparing plasma samples from different type of pituitary adenomas and normal samples. Regarding 5p isomiRs, miR-140-3p showed significant difference in plasma of patients with GHPA compared to normal samples. MiR-1-3p SNP isomiRs showed higher expression in preoperative plasma samples from patients with GHPA compared to FSH/LH+ and HN samples. #6 SNP isomiR of miR-1-3p appeared only in HN samples while it was undetectable

in other groups. Regarding miR-184, significant differences were identified in 3p isomiRs between GH+ and HN plasma samples. Interestingly, #6 isomiR variant of miR-184 was expressed only in HN samples whereas it was unnoticeable in other groups. This variant is shorter at the 3' end and carries 2 SNPs in its sequence. This characteristic read represented 48% of all miR-184 population in HN samples.

Earlier, several reports showed that pituitary and pituitary adenoma tissues are rich in miRNAs. Therefore we cross-checked whether miRNAs differentially expressed between adenoma tissues vs. normal pituitary can be detected in plasma samples.

For this purpose, we collected data from miRNA expression profiling studies available in literature (NFPA: 4, GHPA: 3 studies) and added our current measurements with TLDA as well. We selected miRNAs that showed expressional change of the same direction in at least two studies. In NFPA tissue studies 31 miRNAs fit these criteria. Of these 31 miRNAs, 22 were expressed in plasma samples. 3 (miR-10b-5p, miR-182-5p, miR-26a-2-3p) of the 22 showed the same expressional change in plasma obtained from non-functional (FSH/LH+ and HN) compared to normal plasma and in pre- vs. postoperative plasma samples. In GHPAs, 7 miRNAs changed to the same direction in at least two studies. Of these 5 were expressed in plasma samples. Out of the five miR-29b-3p, miR-432-5p and miR-503-5p showed the same changes in tissues, preoperative plasma vs. normal samples and between pre- and postoperative plasma samples. Common feature of these miRNAs was that they showed extremely low expression level in plasma samples.

To identify potential circulating miRNA biomarkers we analysed whether miRNA profile differs between preoperative and late postoperative plasma samples. We identified 3, 7 and 66 miRNAs in GH+, FSH/LH+ and HN groups that showed different level ($p<0.05$) after pituitary adenoma removal.

To determine the cut-off read number that is possible to be validated by individual TaqMan assay we selected miRNAs in the range of <50, 50-100 and >=100 normalized read numbers. We used the most sensitive RT-qPCR method of which reverse transcription contains preamplification step as well. No amplification by RT-qPCR in the lowest range (miR-6514-3p, miR-6850-5p and miR-6867-5p) was detected.

Next, from significant miRNAs, miRNAs with high coverage for validation were selected (GH+: miR-150-5p, FSH/LH+: miR-143-3p, HN: miR-26b-5p, miR-126-5p, miR-148b-3p). Additionally, discovery set was complemented with additional samples. The decrease of miR-143-3p in postoperative FSH/LH+ samples was confirmed. Although the expression alteration

of miR-26b-5p, miR-126-5p, miR-148b-3p and miR-150-5p by RT-qPCR was similar to NGS results, these changes were not significant ($p>0.05$).

By comparing miR-143-3p expression in preoperative plasma samples from the different histological groups, a significantly higher expression in FSH/LH+ samples was found. In contrast to late postoperative samples, there was no reduction of miR-143-3p expression in early postoperative FSH/LH+ samples (1-3 days after surgery). Plasma miR-143-3p level did not change in GH+, HN and plurihormonal (additional hormone positivity to FSH/LH+) adenoma samples neither in early nor in late postoperative samples compared to their preoperative pairs suggesting its specificity to purely FSH/LH+ adenomas.

By ROC analysis for miR-143-3p level in pre- and late postoperative plasma pairs, the area under curve (AUC) was 0.79 ($p=0.024$) regarding FSH/LH+ adenoma patients. At the cut-off value of -dCT=-5.14 the sensitivity of miR-143-3p expression was 81.8% while the specificity was: 72.7% in discrimination of plasma samples obtained from pre- and late postoperative state of FSH/LH+ pituitary adenoma patients.

Based on our and others previous works it is known that miRNAs in liquid biopsy can be associated with extracellular vesicles (EVs) and potentially serve as more sensitive biomarkers compared to the miRNAs measured from unfractionated plasma. Thus, we separated EVs and analysed our miRNAs. We found that miRNAs undetectable in cell-free plasma (miR-6514-3p, miR-6850-5p and miR-6867-5p) were also not detectable in EVs. All the detectable miRNAs in HN and GH plasma samples (miR-26b-5p, miR-126-5p, miR-148b-3p, miR-150-5p) were measurable in EVs as well, and similarly those did not show significant changes between pre- and postoperative samples. Interestingly, the change of miR-143-3p level in FSH/LH+ samples was also not significant in EVs, suggesting that miR-143-3p is mainly present in plasma associated to protein rather than vesicles.

IV.3. Identification of new therapeutic approaches of pituitary adenomas

Based on TLDA measurement TRAIL was downregulated in NFPA (n=29) compared to NP tissues (n=10) but did not significantly changed in GHPAs (n=12). Survivin was the most highly upregulated gene both in NFPA and GHPA compared to NP tissues, however it was only significant in NFPA tissues by immunohistochemistry. We only observed nuclear but no cytoplasmic localization of survivin protein. Survivin mRNA and protein expression did not show significant correlation with Ki67 index.

As survivin inhibits apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL) and ASA enhances and sensitizes cells to TRAIL-induced apoptosis in different cell lines and

xenograft models, we investigated TRAIL expression in pituitary adenoma. TRAIL was downregulated in non-functioning pituitary adenoma compared to normal pituitary.

RC-4 B/C and GH3 pituitary adenoma cells were resistant to *in vitro* human recombinant TRAIL treatment, and could not be sensitized by acetylsalicylic acid (ASA) pretreatment.

However, ASA significantly decreased cell viability and proliferation in a dose-dependent manner in the RC-4 B/C cell line but not in the growth hormone-secreting GH3 cells, thus we used RC-4 B/C cells in our further expriments. Then we investigated the mechanisms behind the antiproliferative effect of ASA and obtained that apoptosis was not induced, but cell cylcle distribution was changed. Flow cytometry analysis showed increased number of cells in G2/M phase and decreased in S phase in a dose-dependent manner.

We hypothesized that the ASA-related effect on cell proliferation in pituitary adenoma cells might be linked to survivin. We found that 1 mM and 5 mM treatment with ASA gradually decreased survivin mRNA and protein expression in RC-4 B/C cells.

In order to demonstrate that ASA specifically acts through survivin we inhibited survivin using a specific small molecule inhibitor, YM155 as well as silencing with a specific siRNA. We found that both YM155 and survivin siRNA led to dramatic survivin protein decrease and we detected significant reduction of proliferation without any sign of apoptosis induction. Regarding the cell cycle, specific survivin inhibition decreased the number of cells in G2/M phase but in contrast to ASA, did not change the number of cells in S phase. This suggests that ASA has a survivin-independent effect on cell cycle.

Thus we investigated ASA effect on cell cycle regulators and obtained that ASA decreased cyclin A2 (CCNA2), CDK2 an pCDK2 expressions on both mRNA and protein level. However, ASA did not change CDK2/pCDK2 ratio. Taken together, gene expression changes were probably resulted by transcriptional inhibition. We also showed that YM155 and survivin siRNAs did not change CCNA2 and CDK2 expression which confirms that this effect is specific to ASA treatment.

Next, to further investigate survivin's function, we transfected RC-4 B/C cells with a survivin-expressing vector and assessed cell proliferation. However, survivin overexpression did not have any effect on cell proliferation measured by Alamar Blue proliferation assay.

Based on our *in vitro* results we decided to generate a pituitary adenoma animal model using RC-4 B/C cell line to test the effect of per os ASA on tumor formation and tumor growth. RC-4 B/C xenograft has never been generated before, therefore we tried several different conditions. We injected severe combined immunodeficient (SCID) mice with gradually

increasing cell number (5-20x10^6cells/injection). In a preliminary experiment tumor cells were injected in 0.2 ml of growth medium, but tumor formation did not occur in three weeks. Next, we mixed cells with Matrigel before injection, and found that injected cells were palpable after 2x10^7 cells injection only in the first two weeks. However, they were not able to adhere and grow and they absorbed progressively in the following weeks. We used GH3 cells as a positive xenograft control as it has been described in literature. As *in vitro* ASA did not have an effect on GH3 cells we disregarded per os ASA treatment *in vivo*.

V. Conclusions

1. We analysed the whole mitochondrial genome of pituitary adenomas for the first time. We identified 414 novel variants, eight of which were associated with the histological type, and one was found only in non-recurrent adenomas not in the recurrent ones, which suggests an association with less aggressive behaviour. Based on our results mitochondrial variants are not the primary cause of pituitary adenoma formation.
2. The used NGS-based method is an efficient and reliable tool to identify mitochondrial DNA variants.
3. We analysed circulating miRNA and isomiR profiles of pituitary adenoma patients. We showed an overall downregulation of miRNAs compared to healthy individuals. About 46% of identified sequences were isomiRs which indicates their importance. We obtained that miRNAs and isomiRs can both discriminate plasma samples of healthy individuals and patients with different types of pituitary adenomas.
4. The used miRNA sequencing is a sensitive method the results of which have to be validated with the gold standard RT-qPCR technique. We showed that miRNA with low abundance (<50 read) cannot be detected with RT-qPCR, thus cannot be used as biomarkers at the present.
5. Comparing miRNA profiles of pre-, and postoperative plasma pairs we obtained that miR-143-3p can be a potential biomarker of FSH/LH+ non-functioning pituitary adenomas.
6. We showed that *in vitro* acetylsalicylic acid treatment reduces pituitary adenoma cell proliferation through cell cycle inhibition without inducing apoptosis. Behind this antiproliferative effect we showed the relevance of survivin, CCNA2 and CDK2 inhibition. Analysing the effect of YM155 survivin inhibitor we concluded that both acetylsalicylic acid and YM155 are promising therapeutical approaches.

VI. List of publications related to the Ph.D. theses

Németh K, Szücs N, Czirják S, Reiniger L, Szabó B, Barna G, Karászi K, Igaz P, Zivkovic V, Korbonits M, Patócs A, Butz H. (2018) Survivin as a potential therapeutic target of acetylsalicylic acid in pituitary adenomas. Oncotarget 9: 29180–29192.

Németh K, Darvasi O, Likó I, Szücs N, Czirják S, Reiniger L, Szabó B, Kurucz PA, Krokker L, Igaz P, Patócs A, Butz H. (2019) Next-generation sequencing identifies novel mitochondrial variants in pituitary adenomas. J Endocrinol Invest 42: 931-940.

Németh K, Darvasi O, Likó I, Szücs N, Czirják S, Reiniger L, Szabó B, Krokker L, Pállinger É, Igaz P, Patócs A, Butz H. (2019) Comprehensive analysis of circulating microRNAs in plasma of patients with pituitary adenomas J Clin Endocrinol Metab 104: 4151-4168.

Németh K, Darvasi O, Szücs N, Czirják S, Butz H. (2018) [The role of miRNAs in the pathogenesis of pituitary adenomas]. Orv Hetil 159: 252–259.

VII. List of publications not directly linked to the Ph.D. theses

Butz H, **Németh K**, Racz K, Patocs A. (2016) Circulating miRNAs as biomarkers for endocrine disorders. J Endocrinol Invest 39: 1–10.

Patócs A, Igaz P, Tőke J, Lendvai N, Sarkadi B, Grolmusz VK, Butz H, Tóth G, **Németh K**, Gláz E, Kiss R, Pusztai P, Sármán B, Reismann P, Szücs N, Tóth M, Rácz K. (2016) Örökletes phaeochromocytomák és paragangliomák molekuláris genetikai vizsgálatával szerzett hazai tapasztalatok. Magyar Belorvosi Archívum 69: 83–92.

Darvasi O, Szabó PM, **Németh K**, Szabó K, Spisák S, Likó I, Czirják S, Rácz K, Igaz P, Patócs A, Butz H. (2017) Limitations of high throughput methods for miRNA expression profiles in non-functioning pituitary adenomas. Pathol Oncol Res 25: 169-182.

Grolmusz VK, Borka K, Kövesdi A, **Németh K**, Balogh K, Dékány C, Kiss A, Szentpéteri A, Sármán B, Somogyi A, Csajbók É, Valkusz Z, Tóth M, Igaz P, Rácz K, Patócs A. (2017) MEN1 mutations and potentially MEN1-targeting miRNAs are responsible for menin deficiency in sporadic and MEN-1 syndrome associated primary hyperparathyroidism. Virchows Arch 471: 401-411.

Butz H, **Németh K**, Czenke D, Likó I, Czirják S, Zivkovic V, Baghy K, Korbonits M, Kovalszky I, Igaz P, Rácz K, Patócs A. (2017) Systematic Investigation of Expression of G2/M Transition Genes Reveals CDC25 Alteration in Nonfunctioning Pituitary Adenomas. Pathol Oncol Res 23: 633–641.