Final report of research results

The overall objective of this study is to identify novel biomarkers for pituitary adenomas.
In this three year research period we investigated circulating (A), tissue (B) and therapeutical, predictive biomarkers (C) for pituitary adenomas.
As miRNAs are a new class of promising biomarkers and next generation-sequencing (NGS) is a new high-throughput method for quantifying miRNAs, first a complex bioinformatical evaluation was performed comparing various high throughput technologies for miRNA profiling. Here, we established the appropriate technical and bioinformatical approach by determining miRNome of 4 normal pituitary (NP) and 8 NFPA samples on microarray, TaqMan array and next-generation sequencing (1).

A. Evaluation of circulating biomarkers.

Altogether, 146 plasma (preoperative, early- and late postoperative) and extracellular vesicle samples were collected from 44 pituitary adenoma patients (31 gonadotroph, 7 GH-producing and 6 hormone-immunonegative adenomas). Adenomas were classified based on anterior pituitary hormone and transcription factor staining (in line with 2017 WHO Classification of Pituitary Tumors). For miRNA profiling we implemented NGS methodology (using QIAseq miRNA NGS library kit (Qiagen) on Illumina MiSeq instrument. Following NGS individual TaqMan assay was used as validation on extended sample set. Pituitary adenoma tissue miRNAs were also evaluated by TaqMan array and selection of miRNAs was complemented with literature data.

Results:

- ***miRNA profile of preoperative plasma samples.*** 29 miRNAs were revealed which could differentiate plasma samples obtained from patients with different pituitary adenoma subtypes and normal plasma samples using hierarchical cluster analysis.

-***IsomiR analysis in preoperative plasma samples of different adenoma types.*** NGS allows us to detect all miRNA variants (called isomiRs). IsomiR types can be classified as sequence length variants present at miRNA 3' end (iso3p), at 5' end (iso5p) or sequence variants (isoSNP). In our plasma samples 53.8%. of total sequencing reads aligned exactly on miRBase mature miRNA sequences. Accordingly, almost half of the miRNA (46.2%) molecules were present in isomiR form that emphasizes their importance. In our plasma samples only 11 miRNAs with different isomiR variant distribution were identified comparing plasma samples from different type of pituitary adenomas and normal samples.

- ***miRNA expression in preoperative and postoperative samples.*** In order to avoid perioperative effects on miRNA's expression we compared pre- and late postoperative plasma samples (collected after 3 month of pituitary surgery) grouped by different histological types. We identified 3, 7 and 66 differentially expressed miRNA between preoperative and late-postoperative plasma samples in growth hormone-producing (GH), gonadotroph (GO) and hormone negative (HN) groups, respectively.

- ***Pituitary adenoma-specific miRNAs in plasma.*** We cross-checked whether miRNAs differentially expressed between adenoma tissues vs. normal pituitary may be expressed in preoperative vs. normal and preoperative vs. postoperative plasma samples obtained from patients with PA. For this purpose we collected data from miRNA expression profiling studies available in literature where normal tissue was used as reference and we supplemented it with our own dataset obtained by using TaqMan miRNA array cards. We included adenoma tissue miRNAs that showed expressional change of the same direction in at least two studies. We cross-referenced these with miRNAs of same expressional change in plasma obtained from adenoma patients compared to normal plasma and in pre- vs. postoperative plasma samples. Both inHN and GH adenomas 3 miRNAs were identified. Common feature of these miRNAs

was that they showed extremely low expression level in plasma therefore their analysis by routine techniques (ie. real time PCR) would not allow their detection hence they could not serve as biomarkers for pituitary adenomas.

- ***Pituitary-specific miRNAs.*** Investigation of congenital hypopituitarism samples (we used PROP1 gene mutant samples) vs. control plasma we identified 102 miRNAs which did not express in PROP1 mutant samples but expressed in normal plasma samples. These can be potentially pituitary specific miRNAs or miRNAs reflecting pituitary hormone effect. We also identified 16 miRNAs expressing only in PROP1 mutation positive samples but not in normal samples. These microRNAs may reflect the exogenous hormone action. Using differential expression analysis we found 15 significantly differentially expressed miRNAs between normal and PROP1 mutant samples. Of these, 4 showed reasonably high (>50 read numbers; UMI reads) expression level (miR-431-5p: fold change: 0.2, p=0.028; let-7a-5p: fold change: 1.8, p=0.032; miR-370-3p: fold change: 0.2, p=0.036; miR-196b-5p: fold change: 0.2, p=0.038). However, higher sample number would be necessary to increase the validity of these results.

- ***Determination of the cut-off read number that is possible to be validated by individual TaqMan assay***. An intensive validation experiment was carried out in order to evaluate which miRNAs could be validated using quantitative real-time PCR. 4 miRNAs were measured using individual TaqMan Assays in 20 samples (pre- and postoperative samples from patients with gonadotroph adenoma).

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.

Based on these results only the miRNAs showing expression of > 50 UMI reads can be validated by individual TaqMan Assays., hence can be potentially applicable as clinical biomarkers.

-***Individual validation.*** From significant miRNAs revealed byNGS study, miRNAs with high coverage were selected on an extended sample set. MiR-143-3p in GO, miR-26b-5p, miR-126-5p and miR-148b-3p in HN, and miR-150-5p in GH pre- and postoperative samples were determined. The decrease of miR-143-3p in postoperative GO 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.

- ***Investigation of miR-143-3p as potential biomarker for nonfunctional adenomas of gonadotroph origin.*** Comparing expression of miR-143-3p in preoperative plasma samples from the different histological groups a significant higher expression in GO samples was found. In contrast to late postoperative samples there was no reduction of miR-143-3p expression in early postoperative GO 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. Performing ROC analysis for miR-143-3p level in pre- and late postoperative plasma pairs area under curve (AUC) was 0.79 (p=0.024). At the cut-off value -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 GO pituitary adenoma patients.

- ***Investigation of extracellular vesicle-associated miRNAs.*** In the first step extracellular vesicles were characterized. Extracellular vesicles was isolated from plasma samples in 34 blood samples by centrifugation. Then, microvesicle (MV) fraction was analyzed using flow

cytometry antibodies specific to blood cells (platelet: CD62+, CD41, and CD42b, white blood cells: CD45+, MV marker: glycophorin A and apoptotic body marker: AnnexinV). We found that the time between sample collection and surgery had a significant effect on CD62+ MVs (MV number was higher in early-postoperative samples compared to late-postoperative samples). This could be expected as following operation coagulation has a major role in wound healing. Unexpectedly, we could not prove different MV number or MV characteristics between normal vs. preoperative pituitary adenoma or between pre- vs. postoperative samples, as it has been described in other various neoplasms.

In the second step, miRNA content of extracellular vesicles (EVs) was investigated. After confirming the presence of exosomes by flow cytometry and tunable resistive pulse sensing (TRPS) analysis 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. Detectable miRNAs in plasma samples of patients with adenoma (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 GO samples was also not significant in EVs, suggesting that miR-143-3p is mainly present in plasma associated to proteins rather than vesicles.

According to the original work plan the abovementioned results fulfilled ***Objective 1-3*** and the results are included in a submitted manuscript (2).

B. <u>Evaluation of tissue biomarkers.</u>

- ***Investigation of members of cell cycle G2/M transition as potential biomarkers.*** Totally, 80 nonfunctional pituitary adenoma and 14 normal pituitary (NP) tissues were included. Expression of 46 genes encoding members of the G2/M transition was profiled. Both the total and the phospho-CDK1 were overexpressed in adenoma tissues. CDC25A correlated with nuclear localized CDK1 (nCDK1) and with tumor size and nCDK1 with Ki-67 index. Comparing primary vs. recurrent adenomas we found that Ki-67 proliferation index was higher and phospho-CDK1 (inactive form) was downregulated in recurrent tumors compared to primary adenomas. CDC25A targeting miRNAs were downregulated in NFPA and negatively correlated with CDC25A expression (3).
- ***Investigation of mitochondrial variants as tissue biomarkers.*** Our preliminary results (complex integrative bioinformatics analysis by assembling miRNA, transcriptomic and proteomic data of totally 147 NFPA and 56 normal pituitary samples that was referred in the first year report) suggested the possibility of mitochondrial dysfunction in pituitary adenomas, hence we performed mitochondrial genome sequencing experiments on an independent sample set. We analyzed 11 growth hormone (GH) producing and 33 non-functioning (22 gonadotroph (GO) and 11 hormone-immunonegative (HN)) pituitary adenomas using VariantPro™ Mitochondrion Panel on Illumina MiSeq instrument. 496 variants were identified in pituitary adenomas with overall low level of heteroplasmy (7.22%). Samples harboring the highest number of variants had the highest Ki-67 indices independently of histological subtypes. We identified 8 variants (A11251G, T4216C, T16126C, C15452A, T14798C, A188G, G185AG, T16093C) with different prevalence among different histological groups. T16189C was found in 40% of non-recurrent adenomas while it was not present in the recurrent ones (4).
- ***Investigation of miRNAs as tissue biomarkers in spindle-cell oncocytoma (SCO) of the pituitary.*** Due to their rarity (0.1-0.4% of all sellar tumors), little information is available regarding SCO pathogenesis and the miRNA expression profile has not been investigated yet

in this tumor type. Using a total of 9 formalin-fixed paraffin embedded pituitary samples (4 primary oncocytomas, 3 recurrent oncocytomas and 2 normal tissues) we performed NGS for miRNA profiling. We included transcriptome data of additional 6 samples' from NBCI GEO database. Differentially expressed miRNAs in pituitary SCO vs. normal tissue and in recurrent vs. primary SCO were determined. Transcriptome analysis revealed cell cycle alterations while miRNAs influenced mainly metabolic processes (tricarboxylic acid cycle-TCA, carbohydrate, lipid metabolism). Through miRNA-target interaction network the overexpressed Aconitase 2 targeted by two downregulated miRNAs was revealed. Because the functional alteration of ACO2 is well known in pituitary oncocytoma, currently we are performing miRNA-target validation constructing ACO2 3'UTR firefly luciferase plasmid. We aim to co-transfect this construction with miR-744-5p and miR-127-3p miRNA mimics into HeLa cells to validate the mRNA-miRNA interaction.

According to the original work plan the abovementioned results fulfilled ***Objective 4*** and the result are included in a submitted manuscript, which is now under revision (5).

C. <u>Evaluation of therapeutic, predictive biomarkers.</u>

As a potential therapeutical target, survivin was found overexpressed in the majority of pituitary adenomas investigating (66 nonfunctioning (NFPA) and growth hormone (GH)-producing adenomas and 15 normal pituitary samples). It may serve as potential predictive biomarker for acetic-salicylic acid (ASA) treatment.

Using functional assays (cell viability, proliferation, flow cytometry cell cycle analysis, caspase-3 activation and DNA degradation) we found that ASA decreased proliferation but did not induce apoptosis in pituitary cells in vitro. We revealed survivin dependent (by inhibiting survivin) and survivin independent (by inhibiting Cyclin A and CDK2) antineoplastic effect of ASA using pituitary cell lines. As survivin expression decreased upon ASA treatment *in vitro*, it could be suggested as potential predictive biomarker in pituitary adenoma (6).

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