

Evaluation of circulating extracellular vesicle-associated microRNAs in patients with adrenocortical tumors

Ph.D. theses
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I. Introduction

Adrenocortical tumors (ACT) are common in humans, and their prevalence rises with age. The vast majority of these tumors- two thirds of all cases- are represented by hormonally inactive, clinically indolent non-functioning adrenocortical adenomas (NFAs). The infrequent cortisol-producing adenomas (CPAs) result in serious morbidity and mortality associated with Cushing's syndrome. Adrenocortical carcinoma (ACC) is a rare (incidence 0.5-2 cases/million/year), but aggressive neoplasm. The prognosis of ACC is poor as the estimated 5-year survival ranges from 15 to 30 % in advanced stages. There is no reliable preoperative marker for distinguishing ACA from ACC at present. Imaging modalities (CT, MRI) have considerable limitations. Biopsy is not recommended due to difficulties of histological analysis and fear of tumor spread.

The discovery of microRNAs (miRNA, miR) represents one of the most exciting fields of contemporary molecular biology. MiRNAs are short (19-25 nucleotide long) non-protein coding RNA molecules, involved mainly in the post-transcriptional regulation of gene expression. Depending on the degree of complementarity between the miRNA and target mRNA sequences, translational inhibition or mRNA degradation follows. Beside tissue miRNAs, novel studies proved the stable existence of miRNAs in different body fluids, as well.

These blood-borne extracellular miRNAs could serve as minimally invasive biomarkers of malignancy and prognosis in different tumors. miRNAs in the circulation are in part packed in actively secreted extracellular vesicles (EV) (exosomes, microvesicles, apoptotic bodies) that render them very stable, thus can be detected as biomarkers. Several

studies (including ours) demonstrated significantly differential expression of miRNAs in benign and malignant adrenocortical tumors, both in tissue samples and blood. However, the diagnostic sensitivity and specificity were not high enough for clinical applicability. We hypothesize that miRNAs secreted actively in EVs could be more specific minimally invasive biomarkers, than miRNAs isolated from whole plasma. The expression of EV-associated miRNAs and their diagnostic potential in plasma samples of patients suffering from ACT has not been yet compared. Furthermore, the expression of miRNAs in hormonally inactive and cortisol-secreting adrenocortical tumors has not yet been investigated.

II: Aims

The objectives of my Ph.D study were:

1. To investigate the expression of extracellular vesicle (EV)-associated microRNAs (miRNA) and their diagnostic potential in preoperative plasma samples of patients with adrenocortical adenoma (ACA) and adrenocortical carcinoma (ACC).
2. To evaluate the expression of plasma EV-associated miRNAs in patients with non-functioning adrenocortical adenoma (NFA), cortisol-producing adrenocortical adenoma (CPA) and cortisol-producing adrenocortical carcinoma (CP-ACC).
3. To analyze the correlation between cortisol secretion parameters and the expression of EV-associated miRNAs.

III. Materials and Methods

III.1. Patients' and plasma samples

In the first part of my study altogether 46 preoperative plasma samples were investigated. 6 ACA and 6 ACC samples have been included in the screening cohort, whereas 18 ACA and 16 ACC in the validation cohort. In the second part of my study, preoperative plasma EV samples of 13 NFAs, 13 CPAs and 9 CP-ACCs were subjected to EV isolation. We analyzed the correlation between cortisol secretion parameters (basal morning cortisol levels, 24 h urinary free cortisol (UFC) and cortisol after low dose dexamethasone test (LDDT)) and the circulating EV-associated miRNA expression (-dCT). We have also investigated the expression of miRNAs in seven plasma sample pairs before and after LDDT.

III.2. Extracellular vesicle isolation and investigation of EV preparations

We used two different approaches for EV isolation in the first part of my study. First, EVs were isolated with a precipitation method using Total Exosome Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. As a second approach, we isolated EVs with differential centrifugation/ultracentrifugation. In the second part of my study we applied just the kit for EV isolation.

We analyzed the EVs isolated by ultracentrifugation with transmission electron microscopy. We have used a Hitachi 7100 electron microscope (Hitachi Ltd.) for the evaluation of the sections.

We investigated the EVs with flow cytometry, as well. We have adsorbed the EVs onto the surface of formaldehyde/sulfate Latex-Beads (Molecular Probes). CD9, CD63, CD81 and annexin V proteins were investigated. Flow cytometry was performed by FACSCalibur (BD Biosciences). For data analysis, we have used FlowJo Software (Tri Star Inc).

The size distribution of EV-s were characterized by dynamic light scattering (DLS) on a Zetasizer Nano S instrument (Malvern Instrument Ltd.)

III.3. RNA isolation and microRNA expression analyses

III.3.1. EV-associated RNA isolation

Total RNA isolation was performed with Total Exosome RNA and Protein Isolation Kit (Thermo Fisher Scientific). We applied the synthetic spike-in control RNA cel-miR-39 as reference gene.

In case of EVs isolated by differential centrifugation/ultracentrifugation, RNA was isolated from vesicles (n=4 ACA, n=4 ACC) using RNeasy Mini Kit (Qiagen) and RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's protocol. The analysis of vesicular RNA was performed by capillary electrophoresis (Agilent Small RNA Kit) on Agilent 2100 Bioanalyzer (Agilent Technologies).

III.3.2. High-throughput gene and miRNA expression analyses

The EV-associated miRNA expression profiling was performed on TaqMan Human MicroRNA Array A cards v2.0 (Thermo Fisher Scientific). We analyzed the expression of 377 human miRNA in each sample.

The statistical analysis of the raw data was performed by Applied Biosystems qPCR Analysis Modules on Thermo Fisher Cloud software.

III.3.3. Validation of individual miRNAs by quantitative RT-PCR

Based on the result of TaqMan Low Density Array (TLDA) measurements, two miRNAs (hsa-miR-101 and hsa-miR-483-5p) were selected for validation by RT-qPCR on 18 ACA and 16 ACC samples. We used predesigned TaqMan probes for miRNA expression analyses. For the evaluation of the data we used the – dCT method.

The expression of hsa-miR-483-5p in EVs isolated by ultracentrifugation was analyzed as abovementioned. In the second part of my study we investigated the expression of: hsa-miR-22-3p, hsa-miR-27a-3p, hsa-miR-210-3p, hsa-miR-320b and hsa-miR-375.

III.4. Statistical analysis

Statistical analysis of data were performed by GraphPad Prism 7.02 (GraphPad Software). For the identification of differentially expressed miRNAs between ACA and ACC groups, Student's t-test or Mann-Whitney test were used depending on the results of Shapiro-Wilk normality test. In all comparisons p -value < 0.05 was considered statistically significant. To identify miRNA markers applicable for diagnosis, receiver operating characteristics (ROC) analysis was performed by GraphPad.

In the second part of my study for the identification of differentially expressed miRNAs between the three groups, one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis test followed by Dunn test were used based on the results of Shapiro-Wilk normality test. We performed Spearman correlation assay to analyze the correlation of cortisol secretion parameters and miRNA –DCT values. For the evaluation of miRNA expression in response to LDDT, unpaired t-test was performed after Shapiro-Wilk normality test.

IV. Results

IV.1 Characterization of the isolated extracellular vesicles

Transmission electron microscopy (TEM) microphotographs taken from EVs isolated by ultracentrifugation confirmed that the EV size and morphology corresponded to those described for exosomes.

Flow cytometric analysis of EVs isolated by ultracentrifugation confirmed the presence of CD9, CD63, CD81 membrane proteins and annexin V cytosolic protein. In the vesicle preparations isolated by Total Exosome Isolation (from plasma) Kit, we could identify CD9, CD81 and annexin V. Thus we could confirmed the presence of EVs in our samples with TEM and flow cytometry.

We have used the dynamic light scattering technique to measure the size distribution of extracellular vesicles. The vesicles were isolated from 4 samples by the Kit. The Z-average value of the 4 samples was 80.83 ± 19.07 nm.

IV.2. EV-associated miRNA expression profiling in adrenocortical tumors

12 preoperative plasma EV samples (n=6 ACA, n=6 ACC) were first screened to identify differences in circulating EV-associated miRNA expression profiles of ACA and ACC patients. We have found no significant differences in miRNA expression between the two groups. We found two miRNAs (hsa-miR-101 és hsa-miR-483-5p) showing a tendency being different between ACA and ACC samples. These have been selected for further validation.

IV.3. Analysis of miRNA expression by RT-qPCR

IV.3.1. miRNA validation in adrenocortical adenoma and carcinoma by RT-qPCR

We have found significant overexpression of hsa-miR-101 ($p < 0.052$) and hsa-miR-483-5p ($p < 0.0001$) in ACC (n= 16) relative to ACA (n= 18) plasma EV samples.

We have found significantly higher expression of hsa-miR-483-5p ($p = 0.0221$) in EVs isolated by ultracentrifugation, as well. The analysis has been performed on 4 ACA and 4 ACC patients.

IV.3.2. EV-associated miRNA expression analysis in hormonally inactive adenomas and in cortisol-producing adrenocortical tumors

In the second part of my study we analyzed the miRNA expression in preoperative plasma EV samples of 13 NFAs, 13 CPAs and 9 CP-ACCs.

We have observed significant underrepresentation of hsa-miR-22-3p in NFA compared with CPA (fold change (FC)= 3.21; $p < 0.01$), and NFA relative to CP-ACC (FC=7.34; $p < 0.0001$).

Hsa-miR-27a-3p was significantly underrepresented in NFA relative to both CPA (FC=3.55; $p < 0.05$), and CP-ACC (FC=4.59; $p < 0.05$).

We have found significantly higher expression of hsa-miR-320b in CP-ACC (FC=10.88 $p < 0.0001$) and CPA (FC=2.57 $p < 0.05$) relative to NFA. Moreover, the expression of hsa-miR-320b was significantly overrepresented in CP-ACC versus CPA ($p < 0.01$).

Hsa-miR-210-3p was significantly overrepresented only in the CP-ACC versus NFA comparison.(FC=3.1 p<0.05).

Hsa-miR-375 showed no difference among different tumor types.

IV.4. Evaluation of the potential applicability of EV-associated miRNAs as minimally invasive biomarker candidates of adrenocortical malignancy by ROC analysis

In the first part of my study we performed ROC analysis to identify minimally invasive biomarkers applicable for the preoperative diagnosis of ACC. We evaluated miRNAs showing significant overexpression in ACC relative ACA (hsa-miR-101 and hsa-miR-483-5p). $dCT_{\text{hsa-miR-483-5p}}$ relative to cel-miR-39 showed the highest area under curve (AUC) value: 0.965. The sensitivity and the specificity of the test to discriminate ACA and ACC were 87.5 and 94.44, respectively. ROC data of hsa-miR-101 have not yielded promising sensitivity (67.85%) and specificity (83.33%) values

In the second part of my study ROC analysis was performed to test the diagnostic applicability of hsa-miR-320b between CPA and CP-ACC. We have not found high enough sensitivity (88.89) and specificity (76.92) value for clinical purposes. AUC was 0.8632.

IV.5. Analysis the correlation of cortisol secreting parameters to circulating EV-associated miRNA levels

Correlation analyses of cortisol secreting parameters and miRNA –dCT values were performed by Spearman correlation assay. We have not observed any correlation between basal cortisol levels and miRNA expression. However, UFC levels were significantly correlated with circulating hsa-miR-22-3p ($r=0.7606$; $p<0.0001$), hsa-miR-27a-3p ($r=0.7628$; $p<0.0001$) and hsa-miR-320b ($r=0.7843$; $p<0.0001$) expression.

Significant correlation was revealed between cortisol level after LDDT and the expression of hsa-miR-22-3p ($p=0.0237$) and hsa-miR-320b ($p=0.0216$).

IV.6. Evaluation of miRNA expression in response to low dose dexamethasone test

In our previous study, we analyzed the expression of circulating miRNAs following administration of dexamethasone and adrenocorticotropin in individuals with normally functioning hypothalamo-pituitary-adrenal axis.

We have reanalyzed seven sample pairs from our previous study before and after low dose dexamethasone test.

EV-associated hsa-miR-27a-3p has been significantly stimulated by 1 mg dexamethasone, whereas a tendency not reaching significance could be observed for hsa-miR-320b and hsa-miR-22-3p.

V. Conclusions

1.1. We applied a commercially available kit for EV isolation, and could confirm its efficacy. Therefore, we reckon that this methodology can be reliably used for EV isolation and could be easily applicable for clinical purposes. Using flow cytometry, we detected the presence of annexin V, CD9 and CD81 on EVs isolated both by ultracentrifugation and the kit used. The dynamic light scattering measurement confirmed that the size distribution of the isolated EVs corresponded to those described in literature.

1.2. We have found two miRNAs showing a tendency being different between ACA and ACC samples in the screening cohort by applying high-throughput TLDA A-cards. We have confirmed the significant overexpression of hsa-miR-101 and hsa-miR-483-5p in ACC versus ACA samples in the validation cohort. The validation cohort included larger number of patients and was performed by targeted RT-qPCR.

1.3. We have evaluated the potential diagnostical application of EV-associated hsa-miR-483-5p to discriminate ACA and ACC. ROC analysis of data revealed that hsa-miR-483-5p has yielded promising sensitivity (87.5%) and specificity (94.44%). Area under curve was 0.965.

1.4. We confirmed the significantly higher expression of hsa-miR-483-5p in ACC relative to ACA in vesicles isolated by ultracentrifugation, as well.

1.5. EV-associated hsa-miR-483-5p appears to be a promising minimally invasive biomarker in the preoperative diagnosis of ACC.

2.1. We have observed significant overexpression of hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b in both CPA and CP-ACC relative to NFA. Hsa-miR-320b has been significantly overrepresented in CP-ACC compared to CPA. Hsa-miR-210-3p turned out to be significantly overexpressed only in CP-ACC relative to NFA.

2.2. EV-associated miRNAs are differentially expressed in non-functioning benign and cortisol-producing adrenocortical tumors.

3.1. Significant correlation was revealed between circulating miRNA concentrations and urinary free cortisol values for hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b and cortisol after low dose dexamethasone test for hsa-miR-22-3p and hsa-miR-320b. We have observed no correlation of circulating miRNA levels with basal cortisol concentrations.

3.2. EV-associated hsa-miR-27a-3p has been significantly stimulated by 1 mg dexamethasone, whereas a tendency not reaching significance could be observed for hsa-miR-22-3p and hsa-miR-320b in individuals with normally functioning hypothalamo-pituitary-adrenal axis.

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

1. **Perge P**, Butz H, Pezzani R, Bancos I, Nagy Z, Pálóczi K, Nyíró G, Decmann Á, Pap E, Luconi M, Mannelli M, Buzás EI, Tóth M, Boscaro M, Patócs A, Igaz P. (2017) Evaluation and diagnostic potential of circulating extracellular vesicle-associated microRNAs in adrenocortical tumors. *Sci Rep*, 7(1): 5474.
Impact factor 2016: 4.259

2. **Perge P**, Decmann Á, Pezzani R, Bancos I, Fassina A, Luconi M, Canu L, Tóth M, Boscaro M, Patócs A, Igaz P. (2018) Analysis of circulating extracellular vesicle-associated microRNAs in cortisol-producing adrenocortical tumors. *Endocrine*, 59(2): 280–287.
Impact factor 2016: 3.131

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

1. **Perge P**, Decmann A, Igaz P. (2015) A neuroendokrin daganatok kezelése szomatostatinanalógokkal. MAGYAR BELORVOSI ARCHIVUM 68(6): 317-322.
2. **Perge P**, Nagy Z, Igaz I, Igaz P. (2015) Suggested roles for microRNA in tumors. BIOMOLECULAR CONCEPTS 6(1): 149-155.
3. Nagy Z, Baghy K, Hunyadi-Gulyás E, Micsik T, Nyíró G, Rácz G, Butz H, **Perge P**, Kovalszky I, Medzihradzky KF, Rácz K, Patócs A, Igaz P. (2015) Evaluation of 9-cis retinoic acid and mitotane as antitumoral agents in an adrenocortical xenograft model. AMERICAN JOURNAL OF CANCER RESEARCH 5(12): 3645-3658.
Impact factor: 3.425
4. Igaz I, Nyíró G, Nagy Z, Butz H, Nagy Z, **Perge P**, Sahin P, Tóth M, Rácz K, Igaz P, Patócs A. (2015) Analysis of Circulating MicroRNAs In Vivo following Administration of Dexamethasone and Adrenocorticotropin. INTERNATIONAL JOURNAL OF ENDOCRINOLOGY 2015:589230.
Impact factor: 2.376
5. **Perge P**, Nagy Z, Decmann A, Igaz I, Igaz P. (2017) Potential relevance of microRNAs in inter-species

epigenetic communication, and implications for disease pathogenesis. RNA BIOLOGY 14(4): 391-401.

Impact factor 2016: 3.9

6. Nagy Z, Szabó PM, Grolmusz VK, **Perge P**, Igaz I, Patócs A, Igaz P. (2017) MEN1 and microRNAs: The link between sporadic pituitary, parathyroid and adrenocortical tumors? MEDICAL HYPOTHESES 99: 40-44.

Impact factor 2016: 1.066

7. Decmann A, **Perge P**, Nagy Z, Butz H, Patócs A, Igaz P. (2017) Keringő mikroRNS-ek az endokrin daganatok diagnosztikájában. ORVOSI HETILAP 158(13): 483-490.

Impact factor 2016: 0.349

8. Decmann Á, **Perge P**, Tóth M, Igaz P. (2018) Adrenal myelolipoma: a comprehensive review. Endocrine 59(1): 7-15.

Impact factor 2016: 3.131

9. Nagy Z, Decmann A, **Perge P**, Igaz P. (2018) A mikro-RNS-ek patogenetikai és diagnosztikai szerepe mellékvesekéreg-carcinomában ORVOSI HETILAP 159(7): 245-251.

Impact factor 2016: 0.349

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