Analysis of circulating microRNAs in benign and malignant adrenal tumors

PhD thesis

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1. Introduction

Tumors of the adrenal cortex are discovered mostly as incidentalomas. Benign adrenocortical adenoma (ACA) is responsible for 55% of all adrenal incidentalomas. The second most common benign adrenal tumor is the adrenal myelolipoma (AML), while the prevalence of the malignant adrenocortical carcinoma (ACC) is rare, 0.7-2 case per one million population with a very poor prognosis. Differentiation between benign and malignant adrenal tumors is pivotal. For this reason, in the clinical practice, imaging diagnostics are used (CT, MRI). In some cases, the differentiation is very challenging. Circulating *hsa-miR-483-5p* is considered to be the best biomarker differentiating between ACA and ACC, but there is no reliable biomarker in the clinical practice for myelolipomas.

Bilateral adrenal hyperplasia (BAH) and aldosterone-producing adenoma (APA) are the two main forms of primary aldosteronism. The therapy of APA is one-sided adrenalectomy, while BAHs are treated with mineralocorticoid-antagonists. Because of the fundamental difference in the therapy of these diseases, it is very important to differentiate between them. For this purpose, adrenal vein sampling (AVS) is used, which is an invasive method that requires great expertise.

MicroRNAs have been analysed with great effort mostly because of their role in the pathogenesis of diseases. Due to their tissue-specificity, microRNAs are promising biomarkers. Different expressions of microRNAs are

described in various diseases such as tumors. MicroRNAs can be found in tissues as well as in different body fluids. Analysis of blood-borne circulating microRNAs and microRNAs in the urine might contribute to the discovery of minimally invasive or non-invasive biomarkers.

2. Aims

The aims of the studies of my thesis were the following:

a) To evaluate and compare the microRNA expression profiles of adrenal myelolipoma, adrenocortical adenoma and adrenocortical carcinoma both from tissue and plasma samples. To find a tissue and minimally invasive biomarker that is characteristic for adrenal myelolipoma and can differentiate it from adrenocortical carcinoma.
b) To determine and correlate the expressions of plasma and urinary *miR-483-5p* for the evaluation of urinary *miR-483-5p* as a non-invasive biomarker of adrenocortical carcinoma.
c) To determine the microRNA expression profiles of the two main forms of primary aldosteronism (aldosterone-producing adenoma and bilateral adrenal hyperplasia) in order to find a biomarker that can be used to differentiate between the two.

3. Methods

3.1. miRNA expression profiling in adrenal myelolipoma, adrenocortical carcinoma and adrenocortical adenoma

3.1.1. Samples

Tissue and plasma samples of ACA, ACC and AML patients were used. Formalin-fixed paraffin-embedded (FFPE) tissue samples were collected from altogether 71 patients. The discovery cohort contained 30 samples (10 ACA, 10 ACC, 10 AML), and the validation cohort contained 41 independent samples (15 AML, 14 ACC, 10 AML). From 33 independent patients with histologically proven tumors, pre-operative EDTA-anticoagulated plasma samples were collected (11 samples from each group).

3.1.2. Sample processing and miRNA isolation from tissue and plasma samples

From tissue samples, total RNA was isolated by RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific). miRNeasy Serum/Plasma Kit (Qiagen) was used to isolate total RNA from EDTA-anticoagulated plasma samples and 5 µl 5 nM/L *syn-cel-miR-39* was added for purification efficiency. Isolation went always according to the manufacturer's protocol.

3.1.3. Tissue microRNA expression profiling by next-generation sequencing

cDNS was produced from total RNA by QIAseq miRNA Library Kit (Qiagen). cDNS library was prepared for sequencing using MiSeq Reagent Kit v3 (Illumina). Next-generation sequencing was performed on an Illumina MiSeq sequencer. FASTQ files were processed using the online software of Qiagen. Secondary analysis revealed significantly differentially expressed miRNAs between different tumor groups.

3.1.4. Validation of significantly differentially expressed miRNAs

Reverse transcription of total RNA from both tissue and plasma samples was performed using TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific) and individual TaqMan miRNA assays. Individual miRNA assays were: *hsa-miR-451a, hsa-miR-486-5p, hsa-miR-363-3p, hsa-miR-150-5p, hsa-miR-184, hsa-miR-483-5p, hsa-miR-483-3p, hsa-miR-183-3p.* As reference miRNA, for tissue samples *RNU48,* and for plasma samples *cel-miR-39* were used. Quantitative real-time PCR was performed by a Quantstudio 7 Flex Real-Time PCR System and TaqMan Fast Universal PCR Master Mix (2x) (Thermo Fisher Scientific).

3.1.5. Statistical analysis

Statistical power analysis was calculated (Tempest Technologies). Real-time PCR data were processed using GraphPad Prism 7.00 software. For the differentiation between tumor groups, one-way ANOVA or Kruskal-Wallis test was used based on the results of the Shapiro-Wilks normality test. Receiver Operating Characteristic (ROC) analysis was performed if a miRNA showed promising difference between expressions of different tumor groups.

3.1.6. Pathway analysis

Potential target molecules of miRNAs were analysed using Diana mirPAth v.3.

3.2. Comparing plasma and urinary *hsa-miR-483-5p* expressions of ACA and ACC patients

3.2.1. Samples

Samples from altogether 46 patients were analysed. EDTA-anticoagulated and random urine samples were collected from 23 ACA and 23 ACC patients. ACC samples were collected from histologically proven cases. Non-operated ACA diagnoses were based on imaging and patient follow-up.

3.2.2. Sample processing, RNA isolation

From all samples, total RNA was isolated using miRNeasy Serum/Plasma Kit. For purification efficiency, *cel-miR-39* was added.

3.2.3. Determination of *hsa-mir-483-5p* expression by real-time PCR

Reverse transcription of RNAs and quantitative real-time PCR analysis were performed like it is described in chapter **3.1.4.** Expression of *hsa-mir-483-5p* was analysed.

3.2.4. Statistical analysis

Relative miRNA expression (2^{-dCT}) was normalized to urinary creatinine levels in order to align miRNA expression to urine density. To differentiate between ACA and ACC, t-test or Mann-Whitney test was used based on the result of the Shapiro-Wilks normality test. Spearman correlation test was used to evaluate the correlation between plasma and urinary *hsa-miR-483-5p*. Diagnostic applicability of *hsa-miR-483-5p* was evaluated by ROC-analysis.

3.3. Circulating miRNA profiling in primary aldosteronism

3.3.1. Samples

EDTA-anticoagulated plasma samples were collected from altogether 123 patients (61 APA and 62 BAH). The diagnosis of primary aldosteronism was established according to relevant protocols and guidelines. APA and BAH were diagnosed by AVS with or without ACTH stimulation. For the differentiation of APA and BAH, lateralization index (LI) was used. APA samples were collected preoperatively.

3.3.2. Sample processing

Total RNA was isolated from plasma samples like described in chapter **3.2.2.**

3.3.3. miRNA expression profiling from plasma samples by next-generation sequencing

30 samples were subjected to next-generation sequencing (16 APA and 14 BAH). Library preparation and sequencing were performed always according to the manufacturer's protocols. Measurements are described in chapter **3.1.3.**

3.3.4. Validation of individual miRNAs

Significantly differentially expressed miRNAs based on NGS results were subjected to real-time PCR validation on an independent validation cohort of 93 samples. Selected miRNAs were: *hsa-miR-30e-5p, hsa-miR-223-*

3p, *hsa-miR-30d-5p*, *hsa-miR-7-5p*. As reference miRNA, *cel-miR-39* was used. PCR validation was performed according to manufacturer's protocol and measurements are described in chapter **3.1.4.**

3.3.5. Statistical analysis

Statistical power analysis was calculated using statistical power and sample calculator (HyLown Consulting LLC). Real-time PCR data were processed by GraphPad Prism 7.00. Being a multi-center study, comparative statistics were used between same disease groups from different centers, to exclude center-based differences. For this reason, one-way ANOVA or Kruskal-Wallis test was used based on the result of the Shapiro-Wilks normality test. We used standard scores of –dCt values (z-score or z-value: $z = \frac{x-\mu}{\sigma}$, where μ and σ are mean and standard deviation of values from given center, respectively) to eliminate center-related anomalies. For differentiating between APA and BAH groups, t-test or Mann-Whitney-test was used based on the result of the Shapiro-Wilks normality test. Diagnostic applicability of miRNAs was evaluated by ROC-analysis.

4. Results

4.1. miRNA profiling in adrenal myelolipoma, adrenocortical carcinoma and adrenocortical adenoma

4.1.1. Tissue miRNA expression profiling by next-generation sequencing

256 significantly differentially expressed miRNAs were discovered by NGS. In AML - compared to ACA and ACC - significantly upregulated *hsa-miR-451a, hsa-miR-486-5p, hsa-miR-363-3p* and *hsa-miR-150-5p* and in ACC – compared to AML and ACA – significantly upregulated *hsa-miR-483-3p, hsa-miR-184, hsa-miR-483-5p* and *hsa-miR-483-5p* were selected and subjected to PCR validation.

4.1.2. Validation of selected miRNAs

Validation of selected miRNAs was successful on tissue samples. Significant upregulation of *hsa-miR-451a, hsa-miR-486-5p* and *hsa-miR-150-5p* could be confirmed in AML in comparison with ACA and ACC. Significant overexpression of *hsa-miR-184, hsa-miR-483-5p* and *hsa-miR-183-3p* could be confirmed in ACC samples compared to ACA samples but not to AML samples.

4.1.3. Analysing miRNA expression in plasma samples

Hsa-miR-451a and *hsa-miR-363-3p* showed significant overexpression in AML relative to ACA and ACC, and *hsa-miR-486-5p* and *hsa-miR-150-5p* showed significant overexpression in AML only compared to ACC samples. *Hsa-miR-483-5p* and *hsa-miR-483-3p* were significantly upregulated in ACC samples compared to only ACA samples.

4.1.4. Diagnostic applicability of the miRNAs

ROC-analysis revealed the highest area under curve (AUC) values in cases of *has-miR-451a* and *hsa-miR-483-3p* miRNAs. Sensitivity of 90.91% and specificity of 81.82% (cut-off: 3.994) could be achieved when AML was compared to ACC, and sensitivity and specificity of 81.82% (cut-off: 3.676) could be achieved when compared to ACA.

4.1.5. Pathway-analysis

The four significantly overexpressed miRNAs in AML target mRNAs involved in fatty-acid biosynthesis, fatty-acid degradation and fatty-acid metabolism.

4.2. Comparing plasma and urinary *hsa-miR-483-5p* expressions of ACA and ACC patients

4.2.1. Comparison of circulating and urinary *hsa-miR-483-5p* expression in ACA and ACC patients

In concert with previous findings, plasma *hsa-miR-483-5p* was significantly overexpressed in ACC samples compared to ACA samples. In urine samples, *hsa-miR-483-5p* could be detected, but no significant difference in its expression could be demonstrated between ACC and ACA samples. Correlation between circulating and urinary *hsa-miR-483-5p* could not be observed. No significant correlation could be demonstrated between *hsa-miR-483-5p* expression and other laboratory parameters.

4.2.2. Diagnostic applicability of plasma *hsa-miR-483-5p*

ROC-analysis revealed AUC of 0.88, sensitivity of 78.3% and specificity of 87% (cut-off: 12.05) when differentiating between ACC and ACA.

4.3. miRNA expression profiling in primary aldosteronism

4.3.1. miRNA expression profiling in plasma samples by NGS

NGS revealed 50 significantly differentially expressed miRNAs between APA and BAH. According to primary analysis, *hsa-mir-30e-5p, hsa-miR-223-3p, hsa-miR-30d-5p* and *hsa-miR-7-5p* showed the biggest difference between APA and BAH samples.

4.3.2. PCR validation of selected miRNA

Selected miRNAs were validated on an independent validation cohort of 93 samples by RT-qPCR. We could demonstrate significant difference between same disease groups from different centers. However, higher miRNA expression in BAH samples were always observable compared to APA samples. Validation of 3 miRNAs were successful. *Hsa-miR-30e-5p* (p=0.04), *hsa-miR-30d-5p* (p=0.02) and *hsa-miR-7-5p* (p=0.016) were significantly upregulated in BAH samples compared to APA samples. Difference in standard deviations of studied groups was observed: miRNA expression in BAH samples were relatively more homogenous than in APA samples.

4.3.3. Diagnostic applicability of selected miRNAs

Significantly overexpressed miRNAs in BAH showed similar diagnostic applicability: *hsa-miR-7-5p*: AUC: 0.64, sensitivity: 58.7% and specificity: 61.7% (cut-off: 0.13); *hsa-miR-30d-5p:* AUC: 0.64, sensitivity: 58.7% and specificity: 61.7% (cut-off: 0.05); *hsa-miR-30e-5p*: AUC: 0.61, sensitivity: 58.7% and specificity: 61.7% (cut-off: 0.06).

5. Conclusions

1.1. MicroRNA expression of adrenocortical carcinoma, adrenocortical adenoma and adrenal myelolipoma is significantly different. It was demonstrated by NGS and confirmed by RT-qPCR.

1.2. Analysis of circulating miRNAs revealed significant overexpression of *hsa-miR-483-3p* and *hsa-miR-483-5p* in adrenocortical carcinoma compared to adrenocortical adenoma. *Hsa-miR-451a* and *hsa-miR-363-3p* showed significant up-regulation in adrenal myelolipoma relative to adrenocortical carcinoma and adenoma.

1.3. *Hsa-miR-483-5p* was significantly upregulated in adrenocortical carcinoma compared to only adrenocortical adenoma, but not to adrenal myelolipoma. This miRNA is considered, so far, to be the best biomarker of adrenocortical carcinoma, and our result might limit its further clinical use.

1.4. *Hsa-miR-451a* showed significant overexpression in adrenal myelolipoma compared to adrenocortical carcinoma and adenoma. Achieving convincing sensitivity and specificity values, after further validations, this miRNA might be used as a minimally invasive biomarker of adrenal myelolipoma.

2.1. We confirmed the significant up-regulation of circulating *hsa-miR-483-5p* in adrenocortical carcinoma compared to adrenocortical adenoma.

2.2. *Hsa-miR-483-5p* is detectable in the urine, but there is no significant difference in the expressions of adrenocortical carcinoma and adenoma samples. It can be stated that urinary *hsa-miR-483-5p* is not suitable as a non-invasive biomarker of adrenocortical carcinoma.

3.1. Circulating *hsa-miR-7-5p, hsa-miR-30d-5p* and *hsa-miR-30e-5p* showed significant overexpression in bilateral adrenal hyperplasia compared to aldosterone-producing adenoma by both NGS and RT-qPCR. Sensitivity and specificity values are – according to our current knowledge - not high enough to use these miRNAs in the clinical practice.

3.2. miRNA expression in bilateral adrenal hyperplasia was more homogenous than in aldosterone-producing adenoma, which is a genetically more heterogeneous disease according to the literature. Our results might strengthen the hypothesis, that these two forms of primary aldosteronism are members of the same spectrum of disease, but of different severity and manifestation.

6. List of publications related to the PhD thesis

1. Decmann A, Perge P, Tóth M, Igaz P. (2018) Adrenal myelolipoma: a comprehensive review. *Endocrine* 59: 7-15.
   Impact factor (2018): 3.296

2. Decmann A, Perge P, Nyírő G, Darvasi O, Likó I, Borka K, Micsik T, Tóth Z, Bancos I, Pezzani R, Iacobone M, Patocs A, Igaz P. (2018) MicroRNA expression profiling in adrenal myelolipoma. *J Clin Endocrinol Metabol* 103: 3522-3530.
   Impact factor (2018): 5.605

3. Decmann A, Bancos I, Khanna A, Thomas MA, Turai P, Perge P, Pintér JZ, Tóth M, Patócs A, Igaz P. (2019) Comparison of plasma and urinary microRNA-483-5p for the diagnosis of adrenocortical malignancy. *J Biotechnol* 297: 49-53.
   Impact factor (2018): 3.163

7. List of publications not directly linked to the PhD thesis

1. Perge P, Decmann A, Igaz P, (2015). A neuroendokrin daganatok kezelése szomatosztatinanalógokkal. *Magy Belorv Arch* 68: 317-322.

2. Perge P, Butz H, Pezzani R, Bancos I, Nagy Z, Pálóczi K, Nyírő G, Decmann A, Pap E, Luconi M, Mannelli M, Buzas 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: 5474
   Impact factor (2017): 4.122

3. 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 Biol* 14: 391-401.
   Impact factor (2017): 5.216

4. Decmann A, Perge P, Nagy Z, Butz H, Patócs A, Igaz P. (2017) Keringő mikroRNS-ek az endokrin daganatok diagnosztikájában. *Orv Hetil* 158: 483-491.
   Impact factor (2017): 0.322

5. Nagy Z, Decmann A, Perge P, Igaz P. (2018) A mikroRNS-ek patogenetikai és diagnosztikai szerepe mellékvesekéreg-carcinomában. *Orv Hetil* 159: 245-251.
   Impact factor (2018): 0.564

6. Perge P, Decmann A, 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: 280-287.
   Impact factor (2018): 3,296

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