CLOSING REPORT
K115398
Studies on circulating microRNA and extracellular vesicles for the diagnosis of adrenal tumours

PI: Prof. Peter Igaz

The aim of this research project was to study circulating microRNAs and extracellular vesicles as biomarkers of adrenocortical tumours. As the differential diagnosis of adrenocortical tumours is often difficult, such biomarkers could be of invaluable help for the establishment of malignancy, and for the follow-up of these tumours. We have studied circulating microRNAs both from extracellular vesicles and unfractionated plasma samples. The **cumulative impact factor** of studies published with the help of the K115398 grant is **53.328** (from these my first and last-authored publications represent 37.061). Two successfully defended PhD dissertations have been prepared from these studies (Dr. Pál Perge and Dr. Ábel Decmann).

Extracellular vesicle-associated microRNA in adrenocortical tumours:

Adrenocortical adenoma vs. adrenocortical cancer

We have performed the first analysis on plasma extracellular vesicle-associated microRNA expression in patients suffering from adrenocortical tumours. We have first performed a Taqman TLDA card analysis of microRNA expression on 6 adenoma and 6 cancer samples, as screening. 2 microRNAs (miR-483-5p and miR-101) have been significantly overexpressed in adrenocortical cancer. In the validation cohort, 18 adrenocortical adenomas and 16 adrenocortical cancer samples have been included and the overexpression of both microRNAs could be validated by real-time RT-qPCR relative to cel-miR-39 as reference. We have also performed an ultracentrifugation-based protocol including electron microscopy, flow-cytometry and size distribution analysis in addition to the kit used and confirmed that the extracellular vesicles analyzed were mostly exosomes. Receiver operator characteristics of data revealed dCT-miR-483-5p normalized to *cel-miR-39* to have the highest diagnostic accuracy (area under curve 0.965), and the sensitivity and the specificity were 87.5 and 94.44, respectively (**Fig. 1.**). Circulating, extracellular vesicle-associated miR-483-5p is thus a promising biomarker of adrenocortical malignancy. This study has published in the open access journal Scientific Reports (Perge P, Butz H, Pezzani R, Bancos I, Nagy Z, Pálóczi K, Nyírő G, Decmann A, Pap E, Luconi M, Mannelli M, Buzás EI, Tóth M, Boscaro M, Patócs A, **Igaz P**: Evaluation and diagnostic potential of circulating exosomal microRNAs in adrenocortical tumors. SCIENTIFIC REPORTS, 7, paper 5474, 2017. doi:10.1038/s41598-017-05777-0, IF (2016): 4.122).

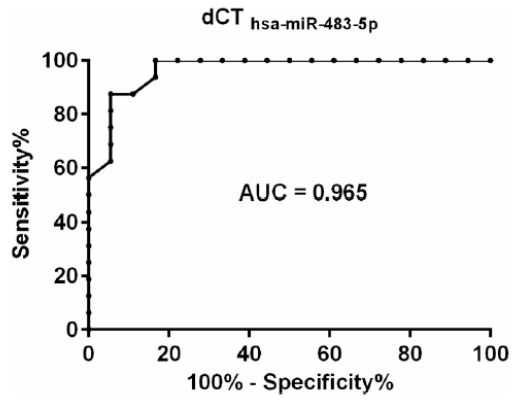

Fig. 1. ROC-curve for extracellular vesicle-associated hsa-miR-483-5p for differentiating adrenocortical cancer from adenoma.

Cortisol-producing adrenocortical adenoma vs. hormonally inactive adrenocortical adenoma

To investigate another aspect of extracellular vesicle-associated circulating microRNAs in adrenocortical tumours, we have compared the expression of selected microRNAs in hormonally inactive adrenocortical adenomas (non-functioning adenoma, NFA) and cortisol-producing adrenocortical adenomas (CPA) and cortisol-producing adrenocortical cancer (CP-ACC) plasma samples. 13 NFAs, 13 CPAs and 9 CP-ACCs were studied for the expression of five microRNAs: *hsa-miR-22-3p, hsa-miR-27a-3p, hsa-miR-210-3p, hsa-miR-320b* and *hsa-miR-375* that were selected based on previous studies. We have observed significant overrepresentation of 3 miRNAs in both CPA and CP-ACC relative to NFA: *hsa-miR-22-3p* ($p<0.01$ and $p<0.0001$, respectively)*, hsa-miR-27a-3p* ($p<0.05$ in both comparisons) and *hsa-miR-320b* ($p<0.05$ and $p<0.0001$, respectively). *Hsa-miR-320b* has been significantly overrepresented in CP-ACC relative to CPA ($p<0.01$). Hsa-miR-210-3p turned out to be significantly overrepresented only in CP-ACC compared to NFA ($p<0.05$). 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* ($p<0.0001$ for all) and cortisol after low dose dexamethasone test for *hsa-miR-22-3p* and *hsa-miR-320b* ($p<0.05$). *Hsa-miR-27a-3p* has been significantly stimulated by low dose dexamethasone test ($p<0.05$). Altogether, we have found circulating extracellular vesicle-associated microRNAs that appear to be related to cortisol overproduction. The gene promoters of 3 of the studied microRNAs contain glucocorticoid responsive elements by *in silico* analysis. The microRNAs might even be implicated in Cushing's syndrome. This work has been published in Endocrine. (Perge P, Decmann A, Pezzani R, Bancos I, Fassina A, Luconi M, Canu L, Tóth M, Boscaro M, Patócs A, Igaz P: Analysis of circulating extracellular vesicle-associated microRNAs in cortisol-producing adrenocortical tumors, Endocrine, 59, 280-287, IF: 3.296)

Circulating microRNAs in other tumours

Circulating microRNAs in adrenal myelolipoma v.s. adrenocortical adenoma and cancer

As the differential diagnosis of adrenal myelolipoma from adrenocortical cancer is sometimes difficult, microRNAs might help to solve this issue. We have first studied tissue microRNAs, and then searched for their circulating blood-borne counterparts for minimally invasive biomarkers applicable in clinical diagnostics. We have screened altogether 30 formalin-fixed paraffin embedded (FFPE) tissue samples (10 adrenocortical cancer (ACC), 10 adrenocortical adenoma (ACA) and 10 adrenal myelolipomas (AML)) by next generation sequencing. By NGS, 256 significantly differentially expressed miRNAs were discovered, and 8 of these were chosen for validation. Validation was performed on 41 independent FFPE samples including 12 ACC, 14 ACA and 15 AML) by real-time RT-qPCR. Significant overexpression of *hsa-miR-451a*, *hsa-miR-486-5p, hsa-miR-363-3p*, and *hsa-miR-150-5p* was confirmed in AML relative to ACA and ACC. *Hsa-miR-184, hsa-miR-483-5p*, and *hsa-miR-183-5p* were significantly overexpressed in ACC relative to ACA but not to AML. Moreover, we have studied altogether 33 plasma samples from patients suffering from ACC, ACA and AML (11 samples from each group) and investigated the microRNAs that are significantly differentially expressed in the tissues. Circulating *hsa-miR-451a* and *hsa-miR-363-3p* were significantly overexpressed in AML, whereas circulating *hsa-miR-483-5p* and *hsa-miR-483-3p* were only significantly overexpressed in ACC vs ACA (**Fig. 2.**). In this study, we used total unfractionated plasma and not extracellular-vesicle preparations, as the latter have not given conclusive results. Based on these findings, we have found both tissue and circulating microRNA markers that can be regarded as specific for adrenal myelolipoma and might help in the differential diagnosis in problematic cases. However, the microRNA considered best for the diagnosis of adrenocortical malignancy, *hsa-miR-483-5p*, has not shown significant differences between AML and ACC, thus this observation might limit its clinical applicability. This study has been published in the leading journal of clinical endocrinology, The Journal of Clinical Endocrinology and Metabolism (Decmann A, Perge P, Nyírő G, Darvasi O, Likó I, Borka K, Micsik T, Tóth Z, Bancos I, Pezzani R, Iacobone M, Patócs A, **Igaz P:** MicroRNA expression profiling in adrenal myelolipoma. J CLIN ENDOCRINOL METAB, 103, 3522-3530, 2018, **IF: 5,605).** This is the first study to report microRNA expression profiling

in adrenal myelolipoma. This ***article was awarded with the ENSAT NAPACA (non-aldosterone producing adrenal adenoma) Prize***

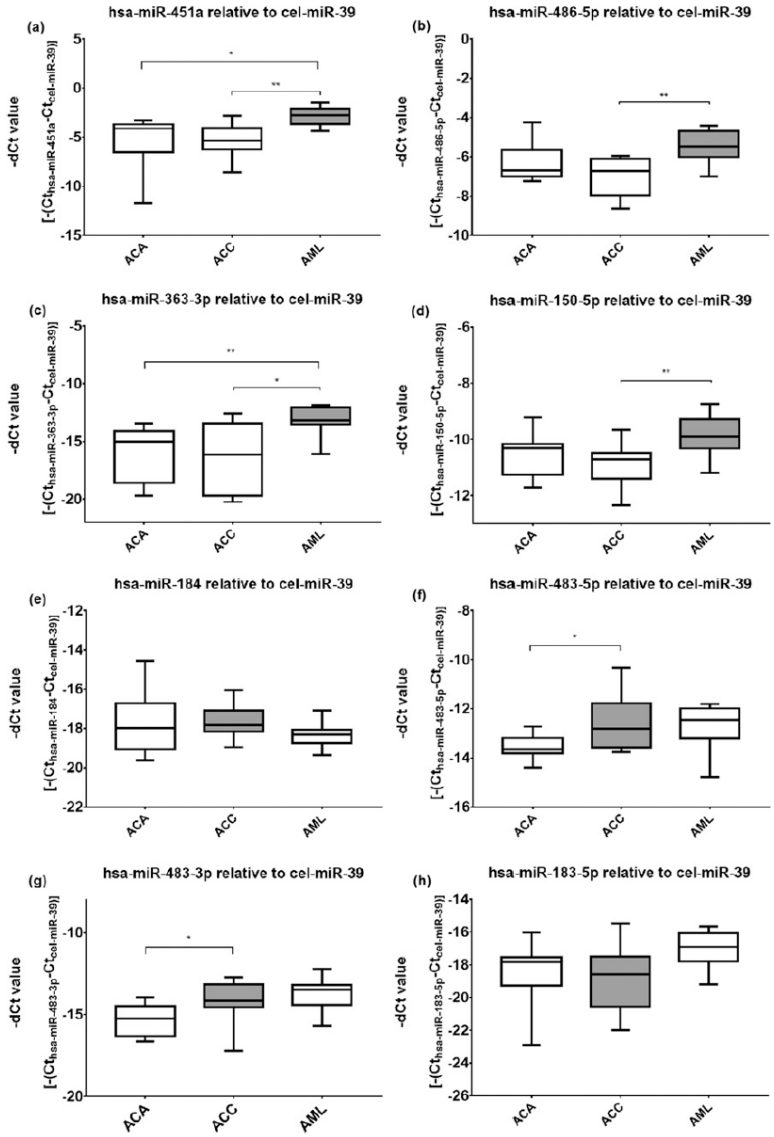

Fig. 2. Circulating miRNA for differentiating adrenocortical adenoma (ACA), adrenocortical cancer (ACC) and adrenal myelolipoma (AML)

Circulating microRNA in primary aldosteronism

As another extension to our original workplan, we have examined the blood-borne circulating microRNAs in patient plasma samples from primary aldosteronism patients. The two major forms of primary aldosteronism i.e. unilateral adenoma (APA) and bilateral adrenal hyperplasia (BAH) can be currently reliable differentiated only by the invasive adrenal venous sampling technique. We first performed NGS on plasma microRNAs from APA and BAH samples (16 APA and 14 BAH). 50 microRNAs turned to show significant differences of expression, and from these, we have selected the four (*miR-30e-5p, miR-30d-5p, miR-223-3p* and *miR-7-5p*) with the highest differences in expression and validated these on an extended cohort of a further 93 plasma samples.. Validation by qRT-PCR confirmed significant overexpression of *hsa-miR-30e-5p, hsa-miR-30d-5p* and *hsa-miR-7-5p* in BAH samples relative to APA (**Fig. 3.**). Regarding the microRNA expressional variations, adenoma is more heterogeneous at the miRNA level compared to hyperplasia. ROC curves, however, have not shown sensitivity-specificity values high enough for consideration in clinical practice, yet. (Abel Decmann, Gábor Nyírő, Ottó Darvasi, Péter Turai, Irina Bancos, Ravinder Jeet Kaur, Raffaele Pezzani, Maurizio Iacobone, Ivana Kraljevic, Darko Kastelan, Mirko Parasiliti-Caprino, Mauro Maccario, Nina Nirschl, Daniel Heinrich, Martin Reincke, Attila Patócs, Peter Igaz: Circulating miRNA expression profiling in primary aldosteronism, Front Endocrinol, 2019, 10, article 739, 2019, IF: 3.634).

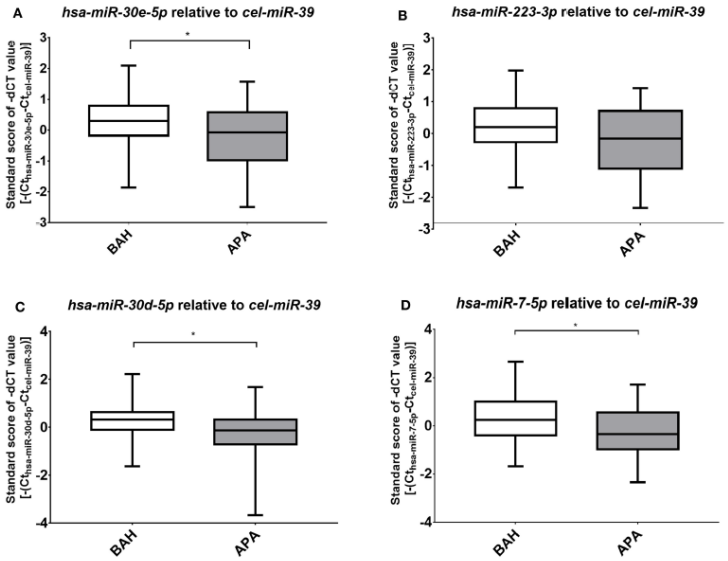

Fig. 3. Validation of 4 miRNA for differentiating unilateral aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH)

Circulating microRNAs for the follow-up of adrenocortical cancer patients

In a collaborative study with the Research Group of Dr. Constanze Hantel at the Ludwig Maximilan University in Munich, we have studied plasma samples of adrenocortical tumor xenograft mice treated with liposomal chemotherapeutic regimens. We have noted no alteration in plasma *miR-483-5p*, but the expression of the major hypoxamir *miR-210* was significantly altered in treated animals. Circulating *miR-210* might thus be exploited as a marker to monitor treatment efficacy in adrenocortical cancer. Manuscript published in Endocrine-Related Cancer (IF: 5.267).

The applicability of circulating microRNAs for adrenocortical cancer therapy monitoring is also supported by our previous xenograft study showing that 9-cis retinoic acid and mitotane in combination significantly suppress adrenocortical xenograft growth. The expression of *miR-483-5p* was significantly suppressed in this model. This study was published in Am J of Cancer Research (IF: 3.425, this study was supported both by my previous NKFIH grant K100295 and the current K115398).

In an on-going study in collaboration with Prof. Alberto Berruti and Dr. Sandra Sigala at the university of Brescia, we are studying adrenocortical cancer patients to examine the utility of *miR-483-5p* and

miR-210 both from unfractionated plasma samples and extracellular vesicles for patient follow-up. If successful, this study could be of great clinical relevance, as there is no reliable marker of adrenocortical cancer follow-up at present.

Urinary microRNA for adrenocortical cancer diagnosis

Although not included in our original workplan, we have also performed a study on the applicability of urinary *hsa-miR-483-5p* as a potential non-invasive marker of adrenocortical malignancy. Whereas we could confirm the overexpression of *hsa-miR-483-5p* in the plasma samples of adrenocortical cancer patients relative to patients having adenomas, there has been no difference in the expression of urinary microRNAs. Urinary *miR-483-5p* is thus unsuitable for the diagnosis of adrenocortical cancer. (Decmann, …, Igaz, J Biotechnology, 2019, IF: 3.163).

I have also participated in a study on extracellular vesicles released from colorectal cancer cells that has been published in J Extracell Vesicles (Valcz et al., J Extracell Ves, 2019, 8:1, 1596668, DOI: 10.1080/20013078.2019.1596668, IF: 11.0)

Review articles published with the support of K115398 Grant

- A review article on the relevance of microRNAs in interspecies communication was published in RNA Biology (Perge P,…, Igaz P, IF: 5.216).
- A review article was published on adrenal myelolipoma in Endocrine (Decmann A, Perge P, Toth M, Igaz P: Adrenal myelolipoma: a comprehensive review, Endocrine, 2018, IF: 3.296)
- Review on circulating microRNAs in adrenal tumours (Igaz P, Curr Opin Endocrinol Diabetes Obes, 2019, IF: 3.298)
- 2 Orvosi Hetilap studies (one awarded with the Markusovszky award)
- 1 manuscript in Medical Hypotheses on the potential relevance of microRNAs in rare endocrine tumors associated with MEN1 syndrome

I would like to thank the support from the National Research, Development and Innovation Office NKFIH that made this intensive research activities possible.

Budapest, 16. November 2019.

Sincerely,

Prof. Peter Igaz MD MSc PhD DSc
Principal Investigator