MicroRNA Expression Profiling in Adrenal Myelolipoma

Abel Decmann,[1] Pál Perge,[1] Gábor Nyírő,[2] Ottó Darvasi,[3] István Likó,[3] Katalin Borka,[4] Tamás Micsik,[5] Zsuzsanna Tóth,[1] Irina Bancos,[6] Raffaele Pezzani,[7] Maurizio Iacobone,[8] Attila Patócs,[3] and Peter Igaz[1,2]

[1]Second Department of Medicine, Faculty of Medicine, Semmelweis University, 1088 Budapest, Hungary; [2]MTA-SE Molecular Medicine Research Group, Hungarian Academy of Sciences and Semmelweis University, 1088 Budapest, Hungary; [3]Hereditary Endocrine Tumors Research Group, Hungarian Academy of Sciences and Semmelweis University, 1088 Budapest, Hungary; [4]Second Department of Pathology, Faculty of Medicine, Semmelweis University, 1091 Budapest, Hungary; [5]First Department of Pathology and Experimental Cancer Research, Faculty of Medicine, Semmelweis University, 1085 Budapest, Hungary; [6]Division of Endocrinology, Diabetes, Metabolism and Nutrition, Department of Internal Medicine, Mayo Clinic, Rochester, Minnesota 55905; [7]Endocrinology Unit, Department of Medicine, University of Padua, 35128 Padova, Italy; and [8]Minimally Invasive Endocrine Surgery Unit, Department of Surgery, Oncology and Gastroenterology (DISCOG), University of Padova, 35128 Padova, Italy

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Introduction: Adrenal myelolipoma (AML) is the second most common and invariably benign primary adrenal neoplasm. Due to the variable proportion of fat and hematopoietic elements and its often large size, it can cause differential diagnostic problems. Several reports confirmed the utility of miRNAs in the diagnosis of tumors, but miRNA expression in AML has not yet been investigated.

Materials and Methods: Next-generation sequencing (NGS) was performed on 30 formalin-fixed, paraffin-embedded (FFPE) archived tissue samples [10 each of AML, adrenocortical adenoma (ACA), and adrenocortical carcinoma (ACC)]. Validation was performed by real-time quantitative reverse transcription polymerase chain reaction on a cohort containing 41 further FFPE samples (15 AML, 14 ACA, and 12 ACC samples). Circulating miRNA counterparts of significantly differentially expressed tissue miRNAs were studied in 33 plasma samples (11 each of ACA, ACC, and AML).

Results: By NGS, 256 significantly differentially expressed miRNAs were discovered, and 8 of these were chosen for validation. 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. 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.

Conclusions: We have found significantly differentially expressed miRNAs in AML and adrenocortical tumors. Circulating *hsa-miR-451a* might be a promising minimally invasive biomarker of AML. The lack of significantly different expression of *hsa-miR-483-3p* and *hsa-miR-483-5p* between AML and ACC might limit their applicability as diagnostic miRNA markers for ACC. ***(J Clin Endocrinol Metab* 103: 3522–3530, 2018)**

Adrenal neoplasms are common. Among adrenal tumors, adrenocortical adenomas (ACAs) are the most frequent, constituting 60% to 70% of adrenal incidentalomas. Adrenal myelolipoma (AML) is the second most common primary adrenal tumor, representing 6% to 16% of all adrenal incidentalomas (1, 2).

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Abbreviations: ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; AML, adrenal myelolipoma; AUC, area under the curve; FC, fold change; FFPE, formalin fixed, paraffin embedded; NGS, next-generation sequencing.

AML is an invariably benign tumor that is composed of adipose tissue and extramedullary hematopoietic elements. The pathogenesis of AML is unclear (3, 4). AMLs are often large tumors with an average size of 10.2 cm at diagnosis (3). On the other hand, adrenocortical carcinoma (ACC) is an uncommon disease with an annual incidence of 0.5 to 2 per million (5–8) and a poor prognosis, with a 5-year survival rate of <15% in stage IV (9, 10).

Because of their large size, it might be occasionally challenging to distinguish AML from other adrenal tumors, especially ACCs, which also often present with a large size (3, 8). Although the presence of macroscopic fat is pathognomonic for AML, the variability in the content of fat and hematopoietic elements in AML could lead to an indeterminate appearance on imaging, and even intense ^{18}F-fluorodeoxyglucose uptake on positron emission tomography–CT due to the hemopoietic elements was reported (11). Moreover, the age distribution of AML is similar to that of ACC, with a peak incidence in the fifth and sixth decades (9).

Mature miRNAs are short, 19- to 25-nucleotide-long single-stranded noncoding RNA molecules that are involved in the regulation of gene expression mostly at the posttranscriptional level. miRNAs are expressed in a tissue-specific fashion and secreted in body fluids (12). Several studies have shown that miRNAs can be useful biomarkers in different diseases, including various neoplasms. Recent studies, including ours, have reported significant differences in tissue and circulating miRNA expression of patients with ACA and ACC (13–17). To our knowledge, the miRNA expression profile of adrenal myelolipoma has not been investigated. With this in mind, we hypothesized that miRNA profiling in AML might lead to the identification of biomarkers that could be used in challenging diagnostic situations.

Materials and Methods

Tissue collection and ethics approval

A total of 71 histologically proven formalin-fixed, paraffin-embedded (FFPE) archived tissue samples were used (Table 1). The discovery cohort contained 30 samples (10 ACA, 10 ACC and 10 AML samples), and the independent validation cohort contained another 41 FFPE samples (15 AML, 14 ACA, and 12 ACC samples). A total of 33 independent preoperative EDTA-anticoagulated plasma samples from patients with histologically proven adrenal tumors (11 samples each of ACA, ACC, and AML) were used for the analysis of circulating miRNA. Preoperative biochemical testing for hormonal evaluation involved basal cortisol, ACTH, aldosterone, renin activity, dehydroepiandrosterone sulfate, urinary catecholamines, and low-dose dexamethasone test (cutoff: 1.8 μg/dL). The study was approved by the Ethical Committee of the Hungarian Health Council. All experiments were performed in accordance with relevant guidelines and regulations, and informed consent was obtained from the involved patients.

Sample processing and RNA isolation

Total RNA was isolated from all the FFPE samples by the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific, Waltham, MA). Total RNA from plasma was isolated by the miRNeasy Serum/Plasma Kit (Qiagen GmbH, Hilden, Germany). As a spike-in control for purification efficiency, 5 μL of 5 nM Syn-cel-miR-39 miScript miRNA Mimic (Qiagen GmbH) was added before the addition of acid-phenol/chloroform. Total RNA was stored at −80°C until further processing.

miRNA expression profiling from tissue samples by next-generation sequencing

The cDNA library was made from total RNA by the QIAseq miRNA Library Kit (Qiagen GmbH) according to the instructions of the manufacturer. The library was prepared for sequencing according to the instructions of the MiSeq Reagent Kit v3 (Illumina, San Diego, CA). Next-generation sequencing (NGS) was performed by Illumina MiSeq (Illumina). FASTQ files were used in the primary data analysis procedure. Qiagen online analysis software was applied. Primary analysis included the trimming of adapters using cutadapt (Marcel Martin, Technical University, Dortmund, Germany). Reads with <16 bp insert sequences or with <10 bp Unique Molecular Index were discarded. Alignment of reads was performed using bowtie (John Hopkins University, Baltimore, MD), and miRbase V21 was used for miRNAs. After DESeq2 normalization (18), secondary analysis revealed significantly differently expressed miRNAs.

Validation of individual miRNAs

RNA was reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific) and individual TaqMan miRNA assays (CN: 4427975; Thermo Fisher Scientific) for tissue and plasma samples. Selected miRNAs were *hsa-miR-451a* (ID: 001141), *hsa-miR-486-5p* (ID: 001278), *hsa-miR-363-3p* (ID: 001271), *hsa-miR-150-5p* (ID: 000473), *hsa-miR-184* (ID: 000485), *hsa-miR-483-5p* (ID: 002338), *hsa-miR-483-3p* (ID: 002339), and *hsa-miR-183-5p* (ID: 002269). The internal control was *RNU48* (ID: 001006) for tissue samples and *cel-miR-39* (ID: 000200) for plasma samples. Quantitative real-time PCR was performed by the TaqMan Fast Universal PCR Master Mix (2x) (CN: 4352042; Thermo Fisher Scientific) on a Quantstudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's protocol for TaqMan miRNA assays with minor modifications. Negative control reactions contained no cDNA templates. Samples were always run in triplicate. For data evaluation, we used the dCt method (delta Ct value equals target miRNA's Ct minus internal control miRNA's Ct) using Microsoft Excel 2016 (Microsoft, Redmond, WA).

Statistical analysis

Statistical power analysis was performed with a statistical power and sample size calculator (Tempest Technologies, Helena, MT). Real-time quantitative PCR data were analyzed by GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA). For differentiating between ACA, ACC, and AML groups, ANOVA or Kruskal-Wallis test was used according to the result

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Table 1. Characteristics of the Tumor and Plasma Samples Studied

Sample	Tumor Type	Cohort	Sample Type	Sex	Age at Sample Taking, y	Hormonal Activity	Tumor Size, mm	Ki-67, %	Weiss Score	ENSAT Stage
1	ACA	Discovery	FFPE	F	55	Nonsecreting	25			
2	ACA	Discovery	FFPE	M	62	Nonsecreting	40			
3	ACA	Discovery	FFPE	M	44	Cortisol	85			
4	ACA	Discovery	FFPE	M	62	Nonsecreting	40			
5	ACA	Discovery	FFPE	M	50	Nonsecreting	40			
6	ACA	Discovery	FFPE	F	57	Nonsecreting	3			
7	ACA	Discovery	FFPE	F	64	Nonsecreting	38			
8	ACA	Discovery	FFPE	M	55	Nonsecreting	60			
9	ACA	Discovery	FFPE	M	44	Cortisol	85			
10	ACA	Discovery	FFPE	F	36	Aldosterone	30			
11	ACC	Discovery	FFPE	F	70	Nonsecreting	120	<20	ND	4
12	ACC	Discovery	FFPE	M	43	Nonsecreting	120	ND	6	4
13	ACC	Discovery	FFPE	F	39	Cortisol	90	10	7	2
14	ACC	Discovery	FFPE	F	58	Nonsecreting	115	ND	ND	2
15	ACC	Discovery	FFPE	F	53	Aldosterone, cortisol	90	40–50	6	2
16	ACC	Discovery	FFPE	F	72	Nonsecreting	10	4–5	ND	3
17	ACC	Discovery	FFPE	F	46	Nonsecreting	200	42	7	4
18	ACC	Discovery	FFPE	F	50	Nonsecreting	ND	ND	5	2
19	ACC	Discovery	FFPE	F	54	Nonsecreting	70	20	3	4
20	ACC	Discovery	FFPE	F	55	Nonsecreting	60	20–30	5	4
21	AML	Discovery	FFPE	M	68	Nonsecreting	80			
22	AML	Discovery	FFPE	F	66	Nonsecreting	70			
23	AML	Discovery	FFPE	F	66	Nonsecreting	35			
24	AML	Discovery	FFPE	F	35	Nonsecreting	60			
25	AML	Discovery	FFPE	F	55	Nonsecreting	60			
26	AML	Discovery	FFPE	F	58	Nonsecreting	80			
27	AML	Discovery	FFPE	M	70	Nonsecreting	90			
28	AML	Discovery	FFPE	F	37	Nonsecreting	80			
29	AML	Discovery	FFPE	M	42	Nonsecreting	80			
30	AML	Discovery	FFPE	M	61	Nonsecreting	50			
31	ACA	Validation	FFPE	F	55	Nonsecreting	90			
32	ACA	Validation	FFPE	M	60	Nonsecreting	30			
33	ACA	Validation	FFPE	F	52	Aldosterone	20			
34	ACA	Validation	FFPE	M	59	Nonsecreting	35			
35	ACA	Validation	FFPE	F	41	Aldosterone	30			
36	ACA	Validation	FFPE	M	51	Aldosterone	45			
37	ACA	Validation	FFPE	F	48	Aldosterone	10			
38	ACA	Validation	FFPE	F	68	Aldosterone	20			
39	ACA	Validation	FFPE	F	43	Aldosterone	15			
40	ACA	Validation	FFPE	F	84	Nonsecreting	90			
41	ACA	Validation	FFPE	F	58	Nonsecreting	40			
42	ACA	Validation	FFPE	F	56	Cortisol	25			
43	ACA	Validation	FFPE	M	25	Nonsecreting	70			
44	ACA	Validation	FFPE	M	64	Nonsecreting	100			
45	ACC	Validation	FFPE	F	57	Nonsecreting	60	20–30	5	4
46	ACC	Validation	FFPE	F	62	Cortisol	78	5	5	2
47	ACC	Validation	FFPE	F	61	Nonsecreting	100	20	5	4
48	ACC	Validation	FFPE	F	48	Nonsecreting	120	30	6	4
49	ACC	Validation	FFPE	F	69	Nonsecreting	110	10–20	6	3
50	ACC	Validation	FFPE	M	25	Cortisol	120	10	6	4
51	ACC	Validation	FFPE	M	79	Nonsecreting	86	10	5	3
52	ACC	Validation	FFPE	F	71	Nonsecreting	80	ND	ND	4
53	ACC	Validation	FFPE	M	17	Nonsecreting	110	10–15	3	2
54	ACC	Validation	FFPE	F	61	Cortisol	80	20–30	5	4
55	ACC	Validation	FFPE	M	28	Nonsecreting	ND	ND	ND	4
56	ACC	Validation	FFPE	F	47	Nonsecreting	140	20–25	4	4
57	AML	Validation	FFPE	F	36	Nonsecreting	100			
58	AML	Validation	FFPE	F	55	Nonsecreting	135			
59	AML	Validation	FFPE	M	51	Nonsecreting	30			
60	AML	Validation	FFPE	F	62	Nonsecreting	40			
61	AML	Validation	FFPE	F	54	Nonsecreting	60			
62	AML	Validation	FFPE	F	35	Nonsecreting	50			
63	AML	Validation	FFPE	M	46	Nonsecreting	60			
64	AML	Validation	FFPE	F	54	Nonsecreting	45			
65	AML	Validation	FFPE	F	38	Nonsecreting	110			
66	AML	Validation	FFPE	M	60	Nonsecreting	80			
67	AML	Validation	FFPE	F	29	Nonsecreting	50			
68	AML	Validation	FFPE	F	42	Nonsecreting	110			
69	AML	Validation	FFPE	F	44	Nonsecreting	40			
70	AML	Validation	FFPE	F	71	Nonsecreting	50			
71	AML	Validation	FFPE	M	60	Nonsecreting	45			
72	ACA	Circulating miRNAs	Plasma	M	62	Cortisol	40			

(*Continued*)

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Table 1. Characteristics of the Tumor and Plasma Samples Studied (Continued)

Sample	Tumor Type	Cohort	Sample Type	Sex	Age at Sample Taking, y	Hormonal Activity	Tumor Size, mm	Ki-67, %	Weiss Score	ENSAT Stage
73	ACA	Circulating miRNAs	Plasma	F	37	Cortisol	51			
74	ACA	Circulating miRNAs	Plasma	F	77	Nonsecreting	75			
75	ACA	Circulating miRNAs	Plasma	M	66	Nonsecreting	50			
76	ACA	Circulating miRNAs	Plasma	M	68	Nonsecreting	50			
77	ACA	Circulating miRNAs	Plasma	F	69	Nonsecreting	35			
78	ACA	Circulating miRNAs	Plasma	F	39	Cortisol	50			
79	ACA	Circulating miRNAs	Plasma	F	22	Cortisol	35			
80	ACA	Circulating miRNAs	Plasma	F	73	Nonsecreting	45			
81	ACA	Circulating miRNAs	Plasma	F	64	Aldosterone	16			
82	ACA	Circulating miRNAs	Plasma	M	47	Aldosterone	12			
83	ACC	Circulating miRNAs	Plasma	F	36	Cortisol	80	ND	ND	4
84	ACC	Circulating miRNAs	Plasma	F	58	Nonsecreting	180	ND	5	4
85	ACC	Circulating miRNAs	Plasma	M	56	Nonsecreting	65	20	5	2
86	ACC	Circulating miRNAs	Plasma	M	39	Cortisol	90	5–10	5	2
87	ACC	Circulating miRNAs	Plasma	M	51	Nonsecreting	170	2	ND	3
88	ACC	Circulating miRNAs	Plasma	M	26	Cortisol	185	10–20	6	3
89	ACC	Circulating miRNAs	Plasma	F	51	Cortisol	10	25	9	3
90	ACC	Circulating miRNAs	Plasma	M	80	Nonsecreting	100	40	10	3
91	ACC	Circulating miRNAs	Plasma	F	56	Testosterone	60	70	6	2
92	ACC	Circulating miRNAs	Plasma	F	62	Testosterone	90	ND	7	3
93	ACC	Circulating miRNAs	Plasma	M	53	Nonsecreting	ND	ND	10	3
94	AML	Circulating miRNAs	Plasma	F	35	Nonsecreting	100			
95	AML	Circulating miRNAs	Plasma	F	59	Nonsecreting	30			
96	AML	Circulating miRNAs	Plasma	F	50	Nonsecreting	45			
97	AML	Circulating miRNAs	Plasma	F	43	Nonsecreting	95			
98	AML	Circulating miRNAs	Plasma	M	39	Nonsecreting	60			
99	AML	Circulating miRNAs	Plasma	M	41	Nonsecreting	110			
100	AML	Circulating miRNAs	Plasma	M	44	Nonsecreting	100			
101	AML	Circulating miRNAs	Plasma	M	49	Nonsecreting	80			
102	AML	Circulating miRNAs	Plasma	M	43	Nonsecreting	120			
103	AML	Circulating miRNAs	Plasma	M	79	Nonsecreting	97			
104	AML	Circulating miRNAs	Plasma	M	42	Nonsecreting	127			

Abbreviations: ENSAT, European Network for the Study of Adrenal Tumors; F, female; M, male; ND, no data.

of the Shapiro-Wilk normality test. miRNAs that could be used potentially as minimally invasive biomarkers in adrenal neoplasms underwent receiver operating characteristic analysis. P values <0.05 were considered significant.

Pathway analysis

The potential targets of miRNAs were investigated using Diana Tools mirPath v.3 (Diana Lab Tools, University of Thessaly, Thessaly, Greece). For target prediction, Targetscan (Whitehead Institute, Cambridge, MA) was used.

Results

miRNA expression profiling by NGS

NGS was performed on 30 FFPE samples. Individual miRNAs are listed in Supplemental Table 1. In total, 256 significantly differentially expressed miRNAs were found. From the top-ranked overexpressed miRNAs in AML listed in Supplemental Table 2, we have selected *hsa-miR-451a* [fold change (FC) to ACC: 14.7; $P < 0.0001$], *hsa-miR-486-5p* (FC: 14.1; $P < 0.0001$), *hsa-miR-363-3p* (FC: 6; $P < 0.0001$), and *hsa-miR-150-5p* (FC: 6.7; $P < 0.0001$) to validate. These miRNAs are significantly upregulated in AML compared with ACA and ACC. *hsa-miR-483-3p* (FC: 47.3; $P < 0.0001$), *hsa-miR-184* (FC: 14.5; $P < 0.0001$), *hsa-miR-483-5p* (FC: 18.2; $P < 0.0001$), and *hsa-miR-183-5p* (FC: 9.5; $P < 0.0001$) were significantly upregulated in ACC compared with AML and ACA (FC and P values compared with AML). NGS data are available under the Gene Expression Omnibus (GEO) accession number GSE112804.

Validation of significantly differentially expressed miRNAs by real-time quantitative reverse transcription polymerase chain reaction

In total, 41 independent FFPE samples were subjected to validation. miRNAs with significantly higher expression in AML relative to ACA and ACC by NGS were successfully validated by real-time quantitative reverse transcription polymerase chain reaction: *hsa-miR-451*, *hsa-miR-486-5p*, *hsa-miR-363-3p*, and *hsa-miR-150-5p* were significantly overexpressed in AML compared with ACA and ACC (Fig. 1). *hsa-miR-363-3p* was significantly overexpressed in AML compared only with ACA, but a tendency of upregulation can be seen relative to ACC.

However, the validation of significantly overexpressed miRNAs in ACC compared with AML and ACA by real-time quantitative PCR was only partly successful, as we could only observe significant overexpression of three miRNAs (*hsa-miR-184*, *hsa-miR-483-5p*, and *hsa-miR-183-5p*) in ACC compared with ACA but not with AML.

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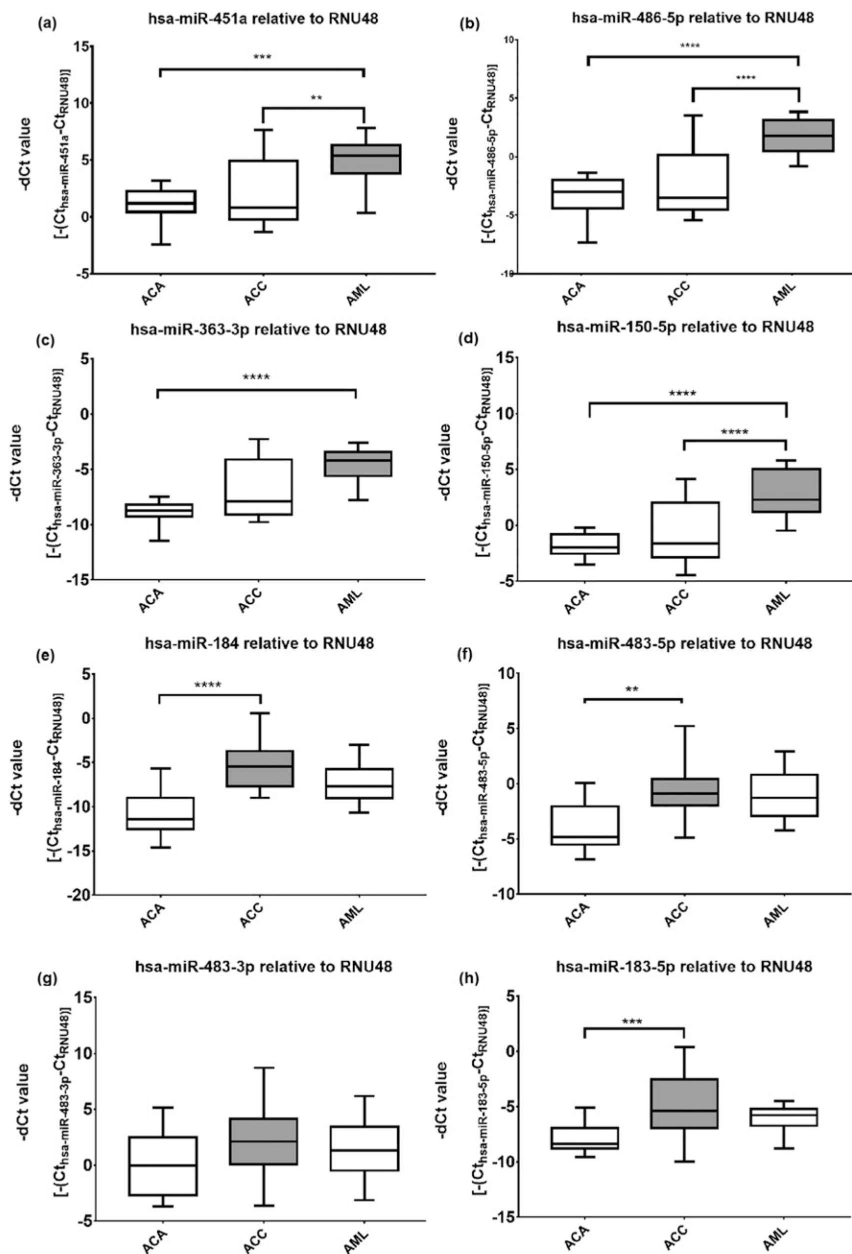

Figure 1. Overexpressed tissue miRNAs in AML and ACC by quantitative reverse transcription polymerase chain reaction. Mean ± SD of −dCt values of selected miRNAs: (a) hsa-miR-451a, (b) hsa-miR-486-5p, (c) hsa-miR-363-3p, (d) hsa-miR-150-5p, (e) hsa-miR-184, (f) hsa-miR-483-5p, (g) hsa-miR-483-3p, and (h) hsa-miR-183-5p. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. ANOVA or Kruskal-Wallis and Tukey or Dunn multiple-comparisons test. Gray shading represents the candidate miRNA.

We have not observed significant differences in the expression of *hsa-miR-483-3p* among the groups studied. Most notably, the expression of *hsa-miR-483-5p* was similar in ACC and AML samples. Statistical power analysis showed that with these 41 samples, the power of our study is >99%.

miRNA expression analysis in plasma samples

Having found significantly differentially expressed miRNAs in tissue samples, we extended our study to plasma samples searching for potential minimally invasive circulating miRNA markers. Significant overexpression of *hsa-miR-451a* and *hsa-miR-363-3p* in AML compared with both ACA and ACC was found (Fig. 2). The expression of *hsa-miR-486-5p* and *hsa-miR-150-5p* was only significantly upregulated in AML compared with ACC but not with ACA.

On the other hand, no significant differences in the expression of *hsa-miR-184* and *hsa-miR-183-5p* were noted. *hsa-miR-483-3p* and *hsa-miR-483-5p* were significantly overexpressed in ACC relative to ACA but not to AML. Statistical power analysis showed that with the 11 samples per group, the power of our study is 0.9985.

Diagnostic performance of miRNAs

Circulating miRNAs that could be potentially used as minimally invasive biomarkers underwent receiver operating characteristic analysis. *hsa-miR-451a* and *hsa-miR-483-3p* showed the highest area under curve (AUC) value. For *hsa-miR-451a*, when AML samples were compared with ACA samples, the AUC was 0.88, and when AML samples were compared with ACC samples, the AUC value was 0.91 (Fig. 3). By selecting 3.676 as the cutoff point, both sensitivity and specificity were 81.82% for differentiating AML and ACA. For differentiating AML and ACC, sensitivity was 90.91% and specificity was 81.82% by setting the cutoff point to 3.994. The negative predictive value of overexpressed *hsa-miR-451a* to rule out ACC was 83.33%, whereas its positive predictive value to confirm AML was 90%.

Circulating *hsa-miR-483-3p* performed best in distinguishing ACC from ACA with an AUC value of 0.88. By setting the cutoff point to 14.42, sensitivity was 81.82%, whereas specificity was 90.91%.

Pathway analysis

Among the predicted targets of *hsa-miR-451a*, *hsa-miR-486-5p*, *hsa-miR-363-3p*, and *hsa-miR-150-5p*, mRNAs coding for proteins involved in fatty acid metabolism, degradation, and biosynthesis were found (3-oxoacyl-ACP synthase, mitochondrial; enoyl-CoA,

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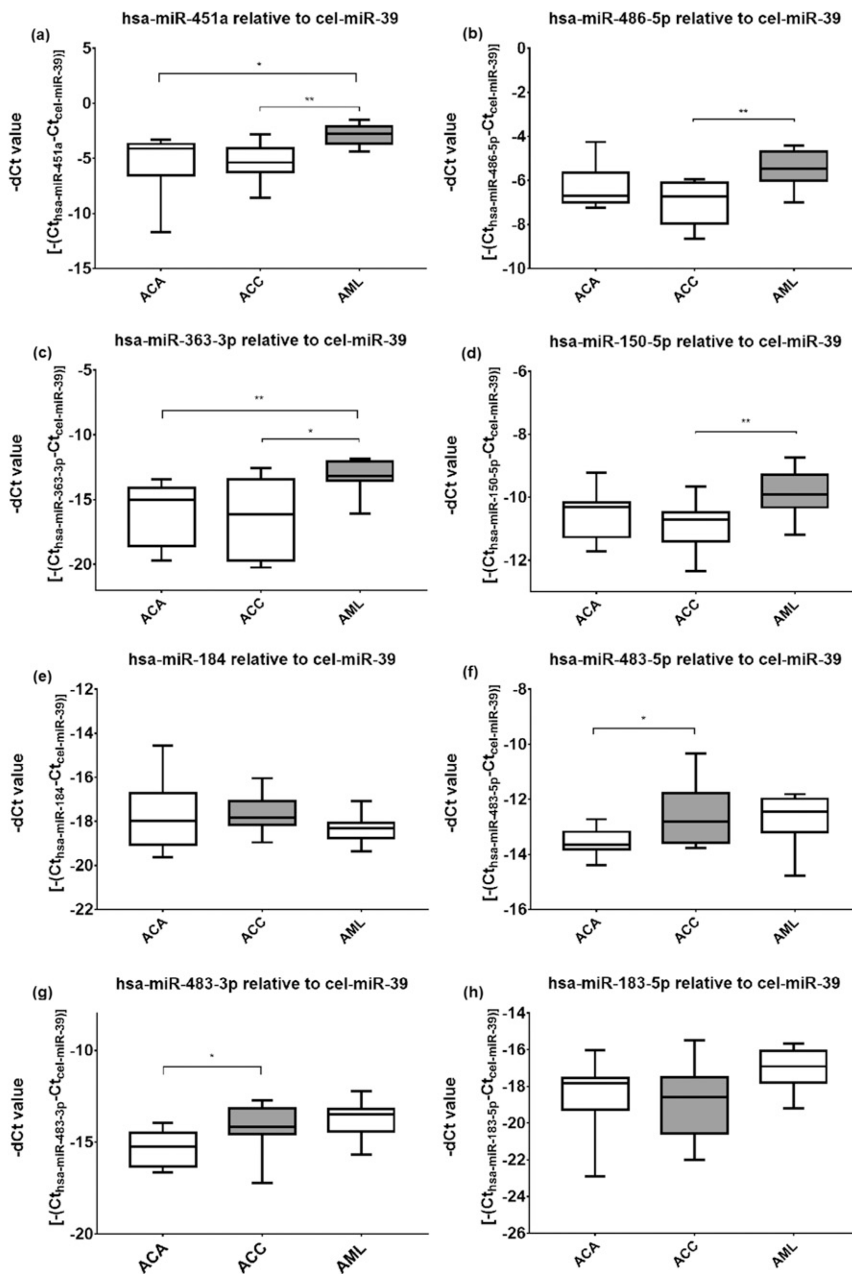

Figure 2. Overexpressed circulating plasma miRNAs in AML and ACC by quantitative reverse transcription polymerase chain reaction. Mean ± SD of −dCt values of selected miRNAs: (a) hsa-miR-451a, (b) hsa-miR-486-5p, (c) hsa-miR-363-3p, (d) hsa-miR-150-5p, (e) hsa-miR-184, (f) hsa-miR-483-5p, (g) hsa-miR-483-3p, and (h) hsa-miR-183-5p. $*P < 0.05$. $**P < 0.01$. ANOVA or Kruskal-Wallis and Tukey or Dunn multiple-comparisons test. Gray shading represents the candidate miRNA.

hydratase/3-hydroxyacyl CoA dehydrogenase; cytochrome P450, family 4, subfamily A, member 22). *P* value was <0.0001 for all the three genes (Table 2).

Discussion

Adrenal myelolipoma is an invariably benign tumor, but it might cause differential diagnostic problems leading to unnecessary procedures. In our study, we have identified miRNA markers specific for AML in tissue and plasma samples. To our knowledge, this is the first report on the miRNA expression profile of AML. Based on the results of NGS, miRNAs *hsa-miR-451a*, *hsa-miR-486-5p*, *hsa-miR-363-3p*, and *hsa-miR-150-5p* performed best in the diagnosis of AML and were able to differentiate AML from ACA and ACC. On the other hand, the already reported ACC-associated miRNAs *hsa-miR-184* (19, 20), *hsa-miR-483-5p* (15, 17), and *hsa-miR-483-3p* were the most highly ranked overexpressed miRNAs in ACC. Overexpression of *hsa-miR-183-5p* has not yet been reported in ACC and represents a novel finding, to our knowledge.

Three of four tissue miRNAs were confirmed by quantitative reverse transcription polymerase chain reaction to be significantly overexpressed in AML relative to ACA and ACC (*hsa-miR-451a*, *hsa-miR-486-5p*, and *hsa-miR-150-5p*). In concert with previous findings (16, 21, 22), we have found that tissue *hsa-miR-483-5p* was significantly overexpressed in ACC relative to ACA, but no difference of expression relative to AML has been observed. Whereas a tendency of *hsa-miR-483-3p* overexpression in ACC was noted, this has not reached statistical significance in our cohort of patients. Overexpression of both *hsa-miR-483-5p* and *hsa-miR-483-3p* has been previously described in ACC (23, 24).

Regarding circulating miRNAs, we demonstrated that *hsa-miR-451a* and *hsa-miR-363-3p* were significantly overexpressed in AML relative to ACA and ACC. In addition, *hsa-miR-486-5p* and *hsa-miR-150-5p* were significantly overexpressed in AML but only compared with ACC and not with ACA. In concordance to previous studies (13, 15, 17, 20), we have observed a significant overexpression of plasma *hsa-miR-483-5p* and *hsa-miR-483-3p* in patients with ACCs, but we could not detect a significant difference of these in expression between AML and ACC.

Tissue and circulating *hsa-miR-483-5p* has been considered the best marker of adrenocortical malignancy to date (13, 15, 17). The noted lack of significance between ACC and AML in the expression of both tissue and

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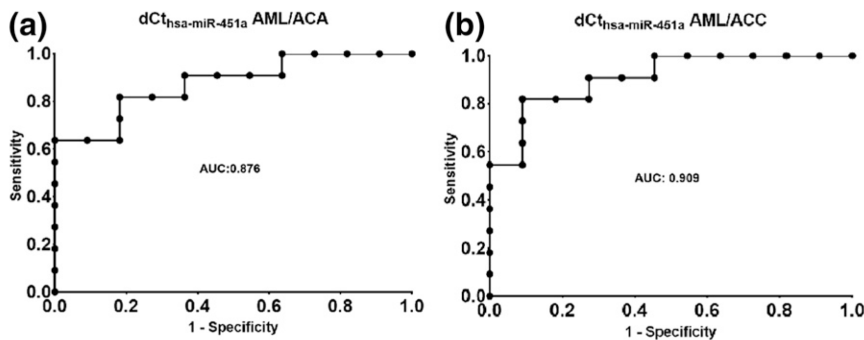

Figure 3. Evaluation of the diagnostic applicability of *hsa-miR-451a* by receiver operating characteristic (ROC) curves. ROC curve of *hsa-miR-451a* in AML compared with ACA and ACC: (a) AML compared with ACA and (b) AML compared with ACC.

plasma *hsa-miR-483-5p* and *hsa-miR-483-3p* is clinically relevant because it might represent a limitation in the use of these markers.

It is intriguing that there has been no significant difference in the tissue expression of *hsa-miR-184*, *hsa-miR-483-3p*, *hsa-miR-483-5p*, and *hsa-miR-183-5p* between ACC and AML, whereas three of these four miRNAs were significantly overexpressed in ACC vs ACA. Although it is pure hypothesis at present, the similar miRNA expression between ACC and AML might indicate some common step in their pathogenesis. Pathway analysis revealed that the significantly overexpressed miRNAs of AML are mostly linked to fatty acid metabolism. The miRNAs overexpressed in AML have been reported to be involved in several tumors. *hsa-miR-451a* was reported to be overexpressed in pancreatic ductal adenocarcinoma (25) and papillary thyroid carcinoma (26) but downregulated in lung adenocarcinoma (27) and melanoma (28). According to the cellular context, the same miRNA can behave as an overexpressed oncogene or downregulated tumor suppressor in different tissues (14). *hsa-miR-486-5p* is mostly downregulated in different tumors and classified as a tumor suppressor (29, 30). Both *hsa-miR-363-3p* (31, 32) and *hsa-miR-150-5p* (33, 34) are mostly downregulated in various tumors. It seems that the overexpressed miRNAs in AML are mostly downregulated in other tumors. AML might thus represent a unique tissue context. Red blood cells are known to harbor *hsa-miR-451* and *hsa-miR-486-5p* (35); moreover, *hsa-miR-451* seems to be involved in erythropoiesis (36). *hsa-miR-451* and *hsa-miR-486-5p* are among the most abundant miRNAs in the blood of healthy individuals (37), and their overexpression in AML might thus be related to the presence of extramedullary hematopoiesis. *hsa-miR-363* that was found to be overexpressed in our AML samples was associated with the regulation of adipogenesis (38).

Tissue and plasma miRNAs are not always parallel. In ACC, for example, tissue *hsa-miR-34a* was downregulated but upregulated in serum samples (15). In another report on endometrioid endometrial carcinoma, the expression of *hsa-miR-9* and *hsa-miR-301b* was differentially expressed in the tissue and in blood (39). Unfortunately, the mechanisms for active miRNA release to body fluids are incompletely understood, and most notably, the processes for miRNA sorting in the extracellular vesicles await clarification (40).

Circulating miRNA markers of AML might be of diagnostic relevance if applied presurgically. Among the miRNAs analyzed, *hsa-miR-451a* appears to be the best candidate for validation studies and possible subsequent integration into clinical practice.

Because ACC is a rare tumor and AML is mostly left nonoperated, the collection of sufficient numbers of preoperative plasma samples from patients with histologically proven tumors is difficult. Whereas we managed to include 25 AML FFPE samples for tissue miRNA analysis, only 11 AML samples for circulating miRNA were available, which is certainly a limitation of this study. Statistical power analysis, however, revealed that the power of our analysis for FFPE and plasma miRNAs has been >99%.

In this study, we have included only samples from patients with a histological diagnosis of adrenal tumors.

Table 2. Results of the Pathway Analysis for miRNA Overexpressed in AML

KEGG Pathway	*P* Value	Gene
Fatty acid metabolism (hsa01212)	<0.0001	OXSM, EHHADH
Fatty acid degradation (hsa00071)	<0.0001	CYP4A22, EHHADH
GABAergic synapse (hsa04727)	<0.0001	SLC38A1, GABRB3, NSF, GABRA4, SLC12A5
Fatty acid biosynthesis (hsa00061)	<0.0001	OXSM
Vitamin B6 metabolism (hsa00750)	<0.001	PNPO

Abbreviations: CYP4A22, cytochrome P450, family 4, subfamily A, member 22; EHHADH, enoyl-CoA and 3-hydroxyacyl CoA dehydrogenase; GABA, γ-aminobutyric acid; GABRA4, γ-aminobutyric acid type A receptor α4 subunit; GABRB3, γ-aminobutyric acid type A receptor β3 subunit; KEGG, Kyoto Encyclopedia of Genes and Ganomes; NSF, *N*-ethylmaleimide sensitive factor, vesicle fusing ATPase; OXSM, 3-oxoacyl-ACP synthase, mitochondrial; PNPO, pyridoxamine 5′-phosphate oxidase; SLC12A5, solute carrier, family 12, member 5; SLC38A1, solute carrier, family 38, member 1.

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However, if the inclusion criteria are less stringent (*i.e.*, plasma samples from patients having AML based on unambiguous imaging diagnosis can be included), the cohorts can be increased considerably. Such a prospective study can be proposed in the future to confirm the utility of AML-associated circulating miRNA markers (mostly circulating *hsa-miR-451a*) as a minimally invasive biomarker. The negative predictive value of overexpressed circulating *hsa-miR-451a* to rule out ACC is not high for clinical introduction at present, but this might be improved by sample size extension in such a further prospective study. Such a marker might be helpful for confirming patients with large tumors to have AML and thus might help to avoid unnecessary surgery.

In conclusion, to our knowledge, we have performed the first miRNA profiling of adrenal myelolipoma and identified miRNAs that are significantly differentially expressed between AML and adrenocortical benign and malignant tumors. Circulating miRNA markers could potentially serve as noninvasive diagnostic biomarkers, but further studies on larger cohorts are needed to confirm their clinical usefulness and applicability.

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Correspondence and Reprint Requests: Peter Igaz, MD, PhD, DSc, Second Department of Medicine, Faculty of Medicine, Semmelweis University, H-1088 Budapest, Szentkirályi str. 46., Hungary. E-mail: igaz.peter@med.semmelweis-univ.hu.

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