MicroRNA Expression Profiling in Adrenal Myelolipoma

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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)
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 <10 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-486-3p (ID: 001278), hsa-miR-363-3p (ID: 001271), hsa-miR-150-3p (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
### 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          |                |          |             |             |
| 2      | ACA        | Discovery  | FFPE        | M   | 62                      | Nonsecreting          |                |          |             |             |
| 3      | ACA        | Discovery  | FFPE        | M   | 44                      | Cortisol              |                |          |             |             |
| 4      | ACA        | Discovery  | FFPE        | M   | 62                      | Nonsecreting          |                |          |             |             |
| 5      | ACA        | Discovery  | FFPE        | M   | 50                      | Nonsecreting          |                |          |             |             |
| 6      | ACA        | Discovery  | FFPE        | F   | 57                      | Nonsecreting          |                |          |             |             |
| 7      | ACA        | Discovery  | FFPE        | M   | 64                      | Nonsecreting          |                |          |             |             |
| 8      | ACA        | Discovery  | FFPE        | M   | 55                      | Nonsecreting          |                |          |             |             |
| 9      | ACA        | Discovery  | FFPE        | M   | 44                      | Cortisol              |                |          |             |             |
| 10     | ACA        | Discovery  | FFPE        | F   | 36                      | Aldosterone           |                |          |             |             |
| 11     | ACC        | Discovery  | FFPE        | F   | 70                      | Nonsecreting          |                |          |             |             |
| 12     | ACC        | Discovery  | FFPE        | M   | 43                      | Nonsecreting          |                |          |             |             |
| 13     | ACC        | Discovery  | FFPE        | F   | 39                      | Cortisol              |                |          |             |             |
| 14     | ACC        | Discovery  | FFPE        | M   | 58                      | Nonsecreting          |                |          |             |             |
| 15     | ACC        | Discovery  | FFPE        | F   | 53                      | Aldosterone, cortisol |                |          |             |             |
| 16     | ACC        | Discovery  | FFPE        | F   | 72                      | Nonsecreting          |                |          |             |             |
| 17     | ACC        | Discovery  | FFPE        | F   | 46                      | Nonsecreting          |                |          |             |             |
| 18     | ACC        | Discovery  | FFPE        | F   | 50                      | Nonsecreting          |                |          |             |             |
| 19     | ACC        | Discovery  | FFPE        | F   | 54                      | Nonsecreting          |                |          |             |             |
| 20     | ACC        | Discovery  | FFPE        | F   | 55                      | Nonsecreting          |                |          |             |             |
| 21     | ACC        | Validation | FFPE        | M   | 68                      | Aldosterone           |                |          |             |             |
| 22     | ACC        | Validation | FFPE        | F   | 66                      | Nonsecreting          |                |          |             |             |
| 23     | ACC        | Validation | FFPE        | F   | 66                      | Nonsecreting          |                |          |             |             |
| 24     | ACC        | Validation | FFPE        | F   | 35                      | Nonsecreting          |                |          |             |             |
| 25     | ACC        | Validation | FFPE        | F   | 55                      | Nonsecreting          |                |          |             |             |
| 26     | ACC        | Validation | FFPE        | F   | 58                      | Nonsecreting          |                |          |             |             |
| 27     | ACC        | Validation | FFPE        | M   | 70                      | Nonsecreting          |                |          |             |             |
| 28     | ACC        | Validation | FFPE        | F   | 37                      | Nonsecreting          |                |          |             |             |
| 29     | ACC        | Validation | FFPE        | M   | 42                      | Nonsecreting          |                |          |             |             |
| 30     | ACC        | Validation | FFPE        | M   | 61                      | Nonsecreting          |                |          |             |             |
| 31     | ACC        | Validation | FFPE        | F   | 55                      | Nonsecreting          |                |          |             |             |
| 32     | ACC        | Validation | FFPE        | M   | 60                      | Nonsecreting          |                |          |             |             |
| 33     | ACC        | Validation | FFPE        | F   | 52                      | Aldosterone           |                |          |             |             |
| 34     | ACC        | Validation | FFPE        | M   | 59                      | Nonsecreting          |                |          |             |             |
| 35     | ACC        | Validation | FFPE        | F   | 41                      | Aldosterone           |                |          |             |             |
| 36     | ACC        | Validation | FFPE        | M   | 51                      | Aldosterone           |                |          |             |             |
| 37     | ACC        | Validation | FFPE        | F   | 48                      | Aldosterone           |                |          |             |             |
| 38     | ACC        | Validation | FFPE        | F   | 68                      | Aldosterone           |                |          |             |             |
| 39     | ACC        | Validation | FFPE        | F   | 43                      | Aldosterone           |                |          |             |             |
| 40     | ACC        | Validation | FFPE        | F   | 84                      | Nonsecreting          |                |          |             |             |
| 41     | ACC        | Validation | FFPE        | F   | 58                      | Nonsecreting          |                |          |             |             |
| 42     | ACC        | Validation | FFPE        | F   | 56                      | Cortisol              |                |          |             |             |
| 43     | ACC        | Validation | FFPE        | M   | 25                      | Nonsecreting          |                |          |             |             |
| 44     | ACC        | Validation | FFPE        | M   | 64                      | Nonsecreting          |                |          |             |             |
| 45     | ACC        | Validation | FFPE        | F   | 57                      | Nonsecreting          |                |          |             |             |
| 46     | ACC        | Validation | FFPE        | F   | 62                      | Cortisol              |                |          |             |             |
| 47     | ACC        | Validation | FFPE        | F   | 61                      | Nonsecreting          |                |          |             |             |
| 48     | ACC        | Validation | FFPE        | F   | 48                      | Nonsecreting          |                |          |             |             |
| 49     | ACC        | Validation | FFPE        | F   | 69                      | Nonsecreting          |                |          |             |             |
| 50     | ACC        | Validation | FFPE        | M   | 25                      | Cortisol              |                |          |             |             |
| 51     | ACC        | Validation | FFPE        | M   | 79                      | Nonsecreting          |                |          |             |             |
| 52     | ACC        | Validation | FFPE        | F   | 71                      | Nonsecreting          |                |          |             |             |
| 53     | ACC        | Validation | FFPE        | M   | 17                      | Nonsecreting          |                |          |             |             |
| 54     | ACC        | Validation | FFPE        | F   | 61                      | Aldosterone           |                |          |             |             |
| 55     | ACC        | Validation | FFPE        | M   | 28                      | Nonsecreting          |                |          |             |             |
| 56     | ACC        | Validation | FFPE        | F   | 47                      | Nonsecreting          |                |          |             |             |
| 57     | AML        | Validation | FFPE        | F   | 36                      | Nonsecreting          |                |          |             |             |
| 58     | AML        | Validation | FFPE        | F   | 55                      | Nonsecreting          |                |          |             |             |
| 59     | AML        | Validation | FFPE        | M   | 51                      | Nonsecreting          |                |          |             |             |
| 60     | AML        | Validation | FFPE        | M   | 62                      | Nonsecreting          |                |          |             |             |
| 61     | AML        | Validation | FFPE        | F   | 54                      | Nonsecreting          |                |          |             |             |
| 62     | AML        | Validation | FFPE        | F   | 35                      | Nonsecreting          |                |          |             |             |
| 63     | AML        | Validation | FFPE        | M   | 46                      | Nonsecreting          |                |          |             |             |
| 64     | AML        | Validation | FFPE        | F   | 54                      | Nonsecreting          |                |          |             |             |
| 65     | AML        | Validation | FFPE        | F   | 38                      | Nonsecreting          |                |          |             |             |
| 66     | AML        | Validation | FFPE        | M   | 60                      | Nonsecreting          |                |          |             |             |
| 67     | AML        | Validation | FFPE        | F   | 29                      | Nonsecreting          |                |          |             |             |
| 68     | AML        | Validation | FFPE        | F   | 42                      | Nonsecreting          |                |          |             |             |
| 69     | AML        | Validation | FFPE        | F   | 44                      | Nonsecreting          |                |          |             |             |
| 70     | AML        | Validation | FFPE        | F   | 71                      | Nonsecreting          |                |          |             |             |
| 71     | AML        | Validation | FFPE        | M   | 60                      | Nonsecreting          |                |          |             |             |
| 72     | ACA        | Circulating miRNAs Plasma | FFPE        | M   | 62                      | Cortisol              |                |          |             |             |

(Continued)
miR-184 (FC: 14.5; and ACC. significantly upregulated in AML compared with ACA (FC: 6.7; miR-363-3p (FC: 6; ACC. miRNAs in AML compared with ACA and ACC (Fig. 1). hsa-miR-363-3p was 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.

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).
We have not observed significant differences in the expression of \textit{hsa-miR-483-3p} among the groups studied. Most notably, the expression of \textit{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 0.999.

**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 \textit{hsa-miR-451a} and \textit{hsa-miR-483-3p} in AML compared with both ACA and ACC was found (Fig. 2). The expression of \textit{hsa-miR-486-5p} and \textit{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 \textit{hsa-miR-184} and \textit{hsa-miR-183-5p} were noted. \textit{hsa-miR-483-3p} and \textit{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. \textit{hsa-miR-451a} and \textit{hsa-miR-483-3p} showed the highest area under curve (AUC) value. For \textit{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 \textit{hsa-miR-451a} to rule out ACC was 83.33%, whereas its positive predictive value to confirm AML was 90%.

Circulating \textit{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 \textit{hsa-miR-451a}, \textit{hsa-miR-486-5p}, \textit{hsa-miR-363-3p}, and \textit{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, 3-oxoacyl-ACP synthase, mitochondrial; enoyl-CoA...
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, hsa-miR-150-5p*). In concert with previous findings (16, 21, 22), we have found that tissue *hsa-miR-363-5p* 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 a 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
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).

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

| KEGG Pathway                        | P Value | Gene                  |
|-------------------------------------|---------|-----------------------|
| Fatty acid metabolism (hsa01212)    | <0.0001 | OXSM, EHHAHDH         |
| Fatty acid degradation (hsa00711)   | <0.0001 | CYP4A22, EHHAHDH      |
| GABAergic synapse (hsa04727)        | <0.0001 | SLC38A1, GABRB3, NSF, GABRA4, SLC12A5 |
| Fatty acid biosynthesis (hsa0061)   | <0.0001 | OXSM                  |
| Vitamin B6 metabolism (hsa00750)    | <0.0001 | PNPO                  |

Abbreviations: CYP4A22, cytochrome P450, family 4, subfamily A, member 22; EHHAHDH, 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 Genomes; 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.

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.
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 has-miR-451a) as a minimally invasive biomarker. The negative predictive value of overexpressed circulating has-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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