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Analysis of circulating microRNAs in adrenocortical tumors

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Differential diagnosis of adrenocortical adenoma (ACA) and carcinoma is of pivotal clinical relevance, as the prognosis and clinical management of benign and malignant adrenocortical tumors (ACTs) is entirely different. Circulating microRNAs (miRNAs) are promising biomarker candidates of malignancy in several tumors; however, there are still numerous technical problems associated with their analysis. The objective of our study was to investigate circulating miRNAs in ACTs and to evaluate their potential applicability as biomarkers of malignancy. We have also addressed technical questions including the choice of profiling and reference gene used. A total of 25 preoperative plasma samples obtained from patients with ACAs and carcinomas were studied by microarray and quantitative real-time PCR. None of the three miRNAs (*hsa-miR-192*, *hsa-mir-197* and *hsa-miR-1281*) found as differentially expressed in plasma samples in our microarray screening could be validated by quantitative real-time PCR. In contrast, of the selected eight miRNAs reported in the literature as differentially expressed in ACT tissues, five (*hsa-miR-100, hsa-miR-181b, hsa-miR-184, hsa-miR-210* and *hsa-miR-483-5p*) showed a statistically significant overexpression in adrenocortical cancer *vs* adenoma when normalized on *hsa-miR-16* as a reference gene. Receiver operator characteristic analysis of data revealed that the combination of $dCT_{hsa\text{-}miR\text{-}210}$ - $dCT_{hsa\text{-}miR\text{-}181b}$ and $dCT_{hsa\text{-}miR\text{-}100}/dCT_{hsa\text{-}miR\text{-}181b}$ showed the highest diagnostic accuracy (area under curve 0.87 and 0.85, respectively). In conclusion, we have found significant differences in expression of circulating miRNAs between ACAs and carcinomas, but their diagnostic accuracy is not yet high enough for clinical application. Further studies on larger cohorts of patients are needed to assess the diagnostic and prognostic potential application of circulating miRNA markers.

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Adrenal tumors are common and their prevalence increases with age reaching 9% in pathological series.[1] The majority of adrenal tumors is of adrenocortical origin (adrenocortical tumors, ACTs), and most of these are benign and hormonally inactive adrenocortical adenomas (ACAs). Hormonally active cortisol- or aldosterone-secreting adenomas are associated with a significant morbidity and mortality.[2] Adrenocortical carcinoma (ACC) is a rare, aggressive tumor with an incidence of 1–2 cases/million people/year and poor prognosis (5-year survival rate around 30%).[3–7]

Differential diagnosis between ACA and ACC is of pivotal clinical relevance, as the prognosis and clinical management of benign and malignant ACTs is entirely different. Imaging techniques including computed tomography, magnetic resonance imaging and positron emission tomography with ^{18}F-2-fluoro-2-deoxy-D-glucose (FDG-PET) can be used for assessing malignancy, but none of these techniques are absolutely reliable.[8,9] It is very difficult to establish malignancy in small adrenal tumors and to exclude it in large tumors with the available imaging techniques. Currently used guidelines propose to remove adrenal tumors with a diameter of >6 cm, as they are associated with a risk of malignancy $>25\%$.[2] Some hormonal features (eg, androgen secretion characteristic for malignant tumors) can also be exploited in

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diagnosis. Most recent data using urinary steroid hormone metabolomics showed characteristic patterns of steroid secretion and metabolism in ACC samples.[10] The histological diagnosis of malignancy is also often difficult[11] and novel markers of malignancy are intensively searched for using bioinformatics approaches to establish an early and specific differential diagnosis between ACC and ACA.

MicroRNAs (miRNAs) are small (~19–24 nucleotide) non-coding RNAs that regulate gene expression at the post-transcriptional level by degrading mRNA molecules or blocking their translation.[12] Numerous studies have shown significantly different tissue miRNA patterns in various neoplasms including ACTs, and some tissue miRNAs can be used for the establishment of malignancy and even as prognostic markers.[13–17] Novel findings revealed that miRNAs can also exist in body fluids and some blood-borne circulating miRNAs have been associated with some diseases including different cancers.[18,19] The source of the circulating miRNAs is unclear, but many miRNAs show the same trend of expression changes in blood samples and tumor tissues of patients with various types of cancer.[12] Recent findings have raised the potential applicability of circulating miRNAs as markers of malignancy in tumors of different organs.[20–24]

Regarding the significant differences in tissue miRNA expression in ACTs reported in several independent studies, we hypothesized that circulating miRNAs might also be differentially expressed in benign and malignant ACTs and might serve as potential minimally invasive biomarkers of malignancy. Technical problems associated with the analysis of circulating miRNAs have also been addressed in our study.

MATERIALS AND METHODS

Patients Samples

Altogether 25 preoperative plasma samples were collected from patients with ACA ($n=12$) and ACC ($n=13$) at Semmelweis University, 2nd Department of Medicine, Hungary and at University of Florence, Italy. The study was approved by the Ethical Committee of the Hungarian Health Council and informed consent was obtained from all patients involved.

In all cases, the diagnosis of ACC was based on the histological examination, whereas the diagnosis of ACA in non-operated cases was based on imaging studies (characteristic cycle threshold (CT) findings and lack of growth during a period of at least 1-year observation). Detailed hormonal profile has been taken in all cases. The characteristics of patients are summarized in Table 1. In all cases, the sampling was performed when the disease was in active phase. Patients did not receive chemotherapy or radiotherapy before blood sampling.

Table 1 Characteristics of patients

Sample number	Disease	Sex	Age at blood taking	Hormonal activity	Ki-67	Weiss score	Tumor stage
1	ACC	F	47	Cortisol	40	8	3
2	ACC	M	46	Cortisol	20	8	4
3	ACC	M	75	Non-secreting	20	5	4
4	ACC	M	26	Cortisol/ androgens	15	7	2
5	ACC	F	58	Androgens	30	7	3
6	ACC	F	58	Androgens	30	7	3
7	ACC	M	18	DHEAS, cortisol	60	6	2
8	ACC	M	57	Non-secreting	5	3	1
9	ACC	F	23	Non-secreting	n.d.	9	4
10	ACC	F	22	Delta-4-androstenedione	15	n.d.	2
11	ACC	F	62	Non-secreting	10	5	2
12	ACC	F	38	Delta-4-androstenedione	1	6	2
13	ACC	F	61	Cortisol	20	8	4
14	ACA	F	35	Non-secreting	n.d.	n.d.	
15	ACA	F	27	Cortisol/ testosterone	n.d.	n.d.	
16	ACA	F	24	Cortisol	n.d.	n.d.	
17	ACA	F	55	Cortisol	n.d.	n.d.	
18	ACA	F	66	Non-secreting	n.d.	n.d.	
19	ACA	F	60	Non-secreting	n.d.	n.d.	
20	ACA	F	79	Non-secreting	n.d.	n.d.	
21	ACA	M	70	Non-secreting	n.d.	n.d.	
22	ACA	F	63	Non-secreting	n.d.	n.d.	
23	ACA	F	62	Non-secreting	n.d.	n.d.	
24	ACA	M	63	Non-secreting	n.d.	n.d.	
25	ACA	M	65	Non-secreting	n.d.	n.d.	

Abbreviation: n.d., not determined.
Tumor stage has been established based on ENS@T (European Network for the Study of Adrenal Tumors) classification.[7]

Sample Processing and Total RNA Isolation

EDTA-anticoagulated blood was collected from patients and processed for plasma isolation immediately after collection. Plasma was obtained by centrifuging whole blood at 3000 r.p.m. for 20 min at 4 °C. All extracted plasma samples were stored in liquid nitrogen until further processing. No hemolysis was observed.

Total RNA was isolated from 200 μl plasma with the Qiagen miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol 'Purification of total RNA, including small RNAs, from serum or plasma using the miRNeasy Mini Kit; with minor modifications. Briefly, plasma was thawed at 37 °C and 200 μl was mixed with 1 ml of QIAzol Lysis Reagent and incubated for 5 min at

room temperature. Then, 5 μl of 5 nM Syn-cel-mir-39 miScript miRNA Mimic (Qiagen GmbH) as a spike-in control for purification efficiency, 2 μl Glycogen (Sigma-Aldrich Chemical Co., St Louis, MO, USA) to enhance the efficiency of RNA column binding and 200 μl chloroform were added, vigorously mixed and incubated for 3 min at room temperature. Samples were centrifuged and the upper aqueous phase was transferred to a collection tube and mixed with 900 μl 100% ethanol. The mixture was applied to RNeasy Mini spin columns and purified according to the protocol. The final total RNA elution volume was 32 μl. RNA concentration was measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in all samples and the concentration of RNA extracted from plasma ranged from 3.3 to 26.9 ng/μl. RNA was stored at −80 °C until use.

MiRNA Expression Profiling

miRNA expression profiling was performed on eight samples (four ACA and four ACC patients) using Affymetrix GeneChip miRNA v 1.0 Array (Affymetrix, Santa Clara, CA, USA) subcontracted by UD-GenoMed Medical Genomics Technologies Ltd (Debrecen, Hungary).

Microarray studies were conducted according to the manufacturer's instructions. Briefly, total RNA (100 ng) was labelled using the FlashTag Biotin HSR Labeling Kit (Genisphere, Hatfield, PA, USA). Hybridization, washing and staining steps were performed using the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The chip was scanned with a GeneChip Scanner 3000 (Affymetrix). All steps were quality controlled by the Affymetrix miRNA QC Tool software. By using spike-in RNA controls, the efficacy of biotinylation and hybridization was also monitored and no significant differences in signal intensity across samples were detected. Results were analyzed using the GeneSpring software 10.1 (Agilent Technologies Inc., Santa Clara, CA, USA) following the instructions of the manufacturer. Robust multi-array (RMA) normalization and baseline transformation at the median of all samples were performed by the GeneSpring software following the Agilent's recommendation.

Raw data were filtered on the level of expression (raw expression is higher than 20 percentile in 100% of samples in at least one group) and fold change (FC) filter was set to 2- or 3-fold between ACA and ACC groups.

Expression of Individual miRNAs by Quantitative Real-Time PCR

Three significantly differentially expressed miRNAs between ACA and ACC based on our microarray screening (*hsa-miR-192-5p*, *hsa-miR-197-3p* and *hsa-miR-1281*) and further eight miRNAs based on the literature research of already reported significantly differentially expressed tissue miRNAs in ACTs (*hsa-miR-100*,[14] *hsa-miR-181b*,[16,17] *hsa-miR-184*,[17] *hsa-miR-195*,[14,16] *hsa-miR-210*,[13,17] *hsa-miR-214*,[14] *hsa-miR-483-5p*,[13,14,16] *hsa-miR-503*[17] were selected for validation.

For reverse transcription quantitative PCR (RT-qPCR) individual Taqman miRNA Assays (Applied Biosystems, Foster City, CA, USA), specific primer/probe combinations provided with each Taqman MicroRNA Assays and Taqman Universal PCR Master mix were used: *hsa-miR-100* (000437), *hsa-miR-181b* (001098), *hsa-miR-184* (000485), *hsa-miR-192-5p* (000491), *hsa-miR-195* (000494), *hsa-miR-197-3p* (000497), *hsa-miR-210* (000512), *hsa-miR-214* (002306), *hsa-miR-483-5p* (002338), *hsa-miR-503* (001048), and *hsa-miR-1281* (241042_mat). To identify the most appropriate reference gene, we also measured the expression level of the most widely used *hsa-mir-16* (000391), *RNU6B* (001093) and *cel-miR-39* (000200).

A fixed volume (5 μl) of total RNA was reverse transcribed with the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) and the specific looped RT primer.[25–27] Quantitative RT-PCR was performed by TaqMan Fast Universal PCR Master Mix (2 ×) (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol with minor modifications (1:10 dilution cDNA and 50 cycles instead of 40 were used).[27] Samples were run in triplicate. Gene expression differences between the various groups were calculated using the dCT (CT) method ($-$dCT values $=-$[CT of target miRNA—CT of internal control miRNA])[28] according to the manufacturer's instructions (SDS Program; Applied Biosystems).

Statistical Analysis

Statistical analysis of microarray data was performed by the GeneSpring 10.1 (Agilent Technologies Inc.) software. For the identification of differentially expressed miRNAs between ACA and ACC groups, unpaired Student's *t*-test followed by Benjamini–Hochberg False Discovery Rate calculation was used.

Statistical analysis of RT-qPCR data was performed by Microsoft Office Excel 2010 (Microsoft Corp., Redmond, WA, USA) and Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) softwares. For the identification of differently expressed genes between ACA and ACC groups after RT-qPCR validation, Student's *t*-test or Mann–Whitney test was used depending on the results of Shapiro–Wilks normality test. To identify miRNA markers applicable for diagnosis, receiver operating characteristics (ROC) analysis was performed by SPSS software version 15 (SPSS 15.0, SPSS Inc., Chicago, IL, USA).

RESULTS

MiRNA Profiling by Microarray

To identify differences in circulating miRNA expression profiles of ACA and ACC patients, eight samples have been subjected to microarray analysis (four ACA and four ACC plasma samples). All results are available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession number: GSE49674.

Of the set of 7815 total miRNA (including 847 human mature miRNAs) transcripts, no significantly differentially expressed miRNAs between ACA and ACC plasma samples have been identified after statistical analysis followed by Benjamini–Hochberg correction. Applying 2-FC filter and unpaired Student's *t*-test without Benjamini–Hochberg correction, two miRNA transcripts, *hsa-miR-192* and *hsa-mir-197*, were found to have a statistically significant differential expression ($P<0.05$) in plasma samples. Both of them were underexpressed in ACC. By applying only the 3-FC filter without statistical analysis, nine differentially expressed human miRNAs were identified: *hsa-miR-24*, *hsa-miR-34b*, *hsa-miR-212*, *hsa-miR-455-3p*, *hsa-miR548a-3p*, *hsa-miR-647*, *hsa-miR-885-5p*, *hsa-miR-923* and *hsa-miR-1281* (Supplementary Table 1).

miRNA Validation by RT-qPCR

To identify the most reliable reference gene to be used for normalization of circulating miRNA in ACA ($n=4$) and ACC ($n=4$) plasma samples analyzed, we tested the most widely used reference genes, such as the spike-in control RNA *cel-miR-39*, the miRNA *hsa-miR-16* and the small nuclear RNA *RNU6B*. After statistical analysis, none of these reference genes showed significant differences between ACA and ACC patient samples. On the basis of the average CT and its standard deviation, *hsa-miR-16* and *cel-miR-39* were more promising candidate reference genes than *RNU6B* (Figure 1). However, we decided to use all three reference genes in our RT-qPCR validation experiments.

On the basis of the microarray results, three miRNAs were selected for further RT-qPCR validation using the same samples as for microarray ($n=4$ ACA and $n=4$ ACC). These included the two significant miRNAs (*hsa-miR-192* and *hsa-miR-197*) found after statistical analysis of microarray data (unpaired *t*-test, FC ≥ 2) and one miRNA (*hsa-miR-1281)* from the set of miRNAs showing FC ≥ 3 without statistical analysis. We have selected the *hsa-miR-1281* because of its considerably high raw expression level in both ACA and ACC, and high FC difference between the two groups. After statistical analysis of RT-qPCR data, none of these miRNAs showed statistically significant differences between ACA and ACC samples based on the average CT and also on dCT approaches compared with all three reference genes studied (data not shown).

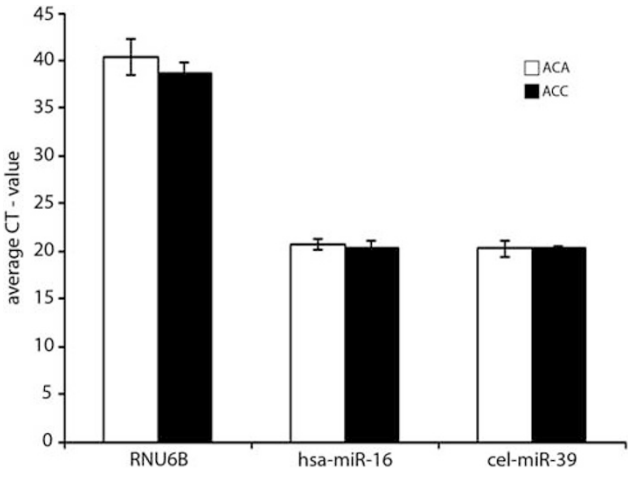

Figure 1 Results of reference gene selection by RT-qPCR. Results are represented by average CT (cycle threshold) (mean ± s.d., $n=4$/group).

On the basis of the literature search, eight miRNAs reported as significantly differentially expressed between ACA and ACC in tissue miRNA studies have been selected for RT-qPCR analysis (ACA $n=8$ and ACC $n=9$): *hsa-miR-100*,[14] *hsa-miR-181b*,[16,17] *hsa-miR-184*,[17] *hsa-miR-195*,[14,16] *hsa-miR-210*,[13,17] *hsa-miR-214*,[14] *hsa-miR-483-5p*,[13,14,16] and *hsa-miR-503*.[17]

In contrast with our microarray data, expression of *RNU6B* seemed to be unstable by RT-qPCR, as in three of eight ACA and in two of nine ACC samples (altogether 29%) no *RNU6B* signal has been observed. By applying *cel-miR-39* as a reference gene, we have found the significantly higher expression of *hsa-miR-181b* in ACC relative to ACA plasma samples (Figure 2a). By using *hsa-miR-16* as a reference gene, *hsa-miR-100*, *hsa-miR-181b*, *hsa-miR-184*, *hsa-miR-210* and *hsa-miR-483-5p* miRNAs have been identified as significantly differentially expressed between ACA and ACC samples. All of these miRNAs were overexpressed in ACC as compared with ACA (Figures 2b–f). By RT-qPCR analysis, we have not found significant differences in expression of *hsa-miR-195*, *hsa-miR-214* and *hsa-miR-503* between ACA and ACC plasma samples. By using the geometric mean of *hsa-miR-16* and *cel-miR-39*, two miRNAs: *hsa-miR-181b* and *hsa-miR-483-5p* have been established as significantly differentially expressed (Figure 3).

Diagnostic Performance of miRNA

To identify potentially applicable miRNA circulating biomarkers of adrenal malignancy, miRNAs with the most significant dCT difference between ACA and ACC (*hsa-miR-100*, *hsa-miR-181b*, *hsa-miR-184*, *hsa-miR-210* and *hsa-miR-483-5p*) and the combination of these miRNA pairs relative to *hsa-miR-16* were tested by ROC analysis. The $dCT_{hsa\text{-}miR\text{-}210}$ -$dCT_{hsa\text{-}miR\text{-}181b}$ and the $dCT_{hsa\text{-}miR\text{-}100}$/$dCT_{hsa\text{-}miR\text{-}181b}$ pairs showed the highest area under curve (AUC) values, 0.87 and 0.85, respectively. By setting the cutoff point of $dCT_{hsa\text{-}miR\text{-}210}$-$dCT_{hsa\text{-}miR\text{-}181b}$ to 2.4, the sensitivity and the specificity of the test to discriminate between ACA and ACC was 88.9 and 75.0%, respectively. Using a cutoff value of 1.69 for the test based on the other pair of miRNA, $dCT_{hsa\text{-}miR\text{-}100}$/$dCT_{hsa\text{-}miR\text{-}181b,}$ the sensitivity and the specificity were 77.8 and 100%, respectively (Figure 4).

DISCUSSION

Circulating miRNAs are promising, minimally invasive biomarker candidates of malignancy in several tumors.[18,19] Regarding the significant differences in the expression of tissue miRNAs in ACTs,[13–17] we aimed to investigate their

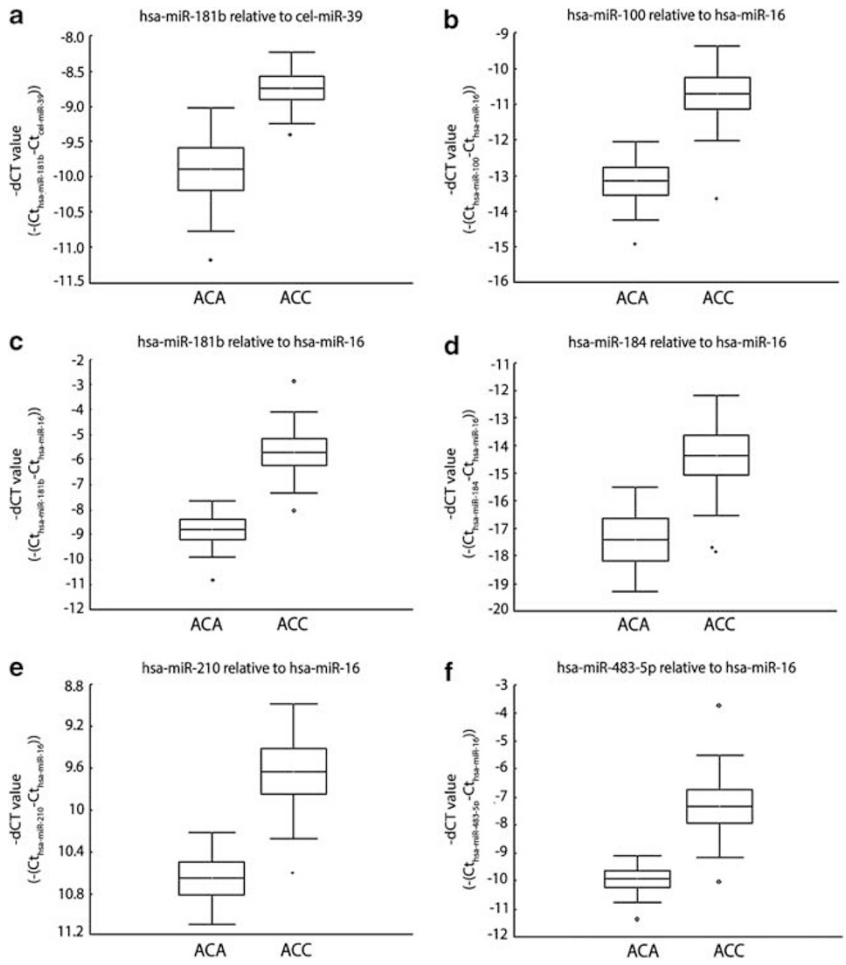

Figure 2 Results of RT-qPCR validation of *hsa-miR-181b* normalized to the housekeeping *cel-miR-39* (**a**) and of *hsa-miR-100* (**b**), *hsa-miR-181b* (**c**), *hsa-miR-184* (**d**), *hsa-miR-210* (**e**) and *hsa-miR-483-5p* (**f**) miRNAs normalized to the reference gene *hsa-miR-16*. Results are represented by $-$dCT (cycle threshold) (mean ± s.d., $P<0.05$; $n=8$ ACA, $n=9$ ACC).

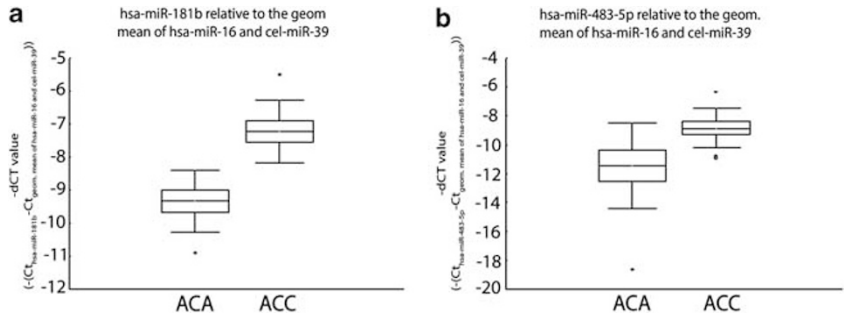

Figure 3 Results of RT-qPCR validation of *hsa-miR-181b* (**a**) and *hsa-miR-483-5p* (**b**) normalized to the geometric mean of two reference genes (*hsa-miR-16* and *cel-miR-39*) (mean ± s.d., $P<0.05$; $n=8$ ACA, $n=9$ ACC).

circulating counterparts and to evaluate their potential applicability as biomarkers. The biogenesis and biological relevance of circulating miRNAs are unclear[12] and there are several unresolved issues in their analysis and study design.

In this study, we have examined the expression of miRNAs in plasma samples of patients suffering from benign and malignant ACTs. We have used both microarray and RT-qPCR for the analysis, and have found some miRNAs by the latter approach that could be useful for differentiating benign and malignant tumors.

One of the most important analytical challenges related to circulating miRNAs is the small amount of miRNA in the

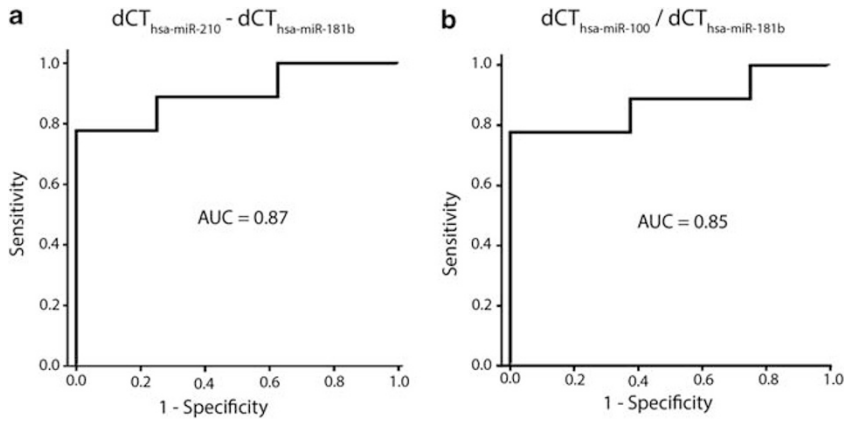

Figure 4 Receiver operating characteristics (ROC) curve analysis for the $dCT_{hsa\text{-}miR\text{-}210}$-$dCT_{hsa\text{-}miR\text{-}181b}$ (**a**) and the $dCT_{hsa\text{-}miR\text{-}100}$/$dCT_{hsa\text{-}miR\text{-}181b}$ (**b**) plasma miRNA signatures relative to the reference gene *hsa-miR-16* according to the results obtained from RT-qPCR analysis of ACA ($n = 8$) and ACC ($n = 9$) plasma samples.

bloodstream. Another major challenge is represented by the difficulties of RNA isolation due to the high protein content of the blood and the contamination by phenol residues that complicate the extraction.[29,30] The major issue to be addressed is the lack of standards that raises problems in raw data normalization.[30] Since generally accepted reference genes have not yet been defined for plasma miRNA, several normalization strategies have been reported.[31] Some studies reported the use of stably expressed genes as a reference, based on earlier investigations (*hsa-miR-16*, *RNU6B* and *RNU48*). However, a constant expression of these genes cannot be hypothesized in all study conditions. Moreover, previous reports described *RNU6B* as having unstable expression and tendency to degradation in plasma/serum samples.[18,21] Indeed, in our study, we also observed *RNU6B* instability in several samples. Other studies applied synthetic exogenous RNA molecules (*cel-miR-39*, *cel-miR-54* and *cel-miR-238*) as reference genes. It is of interest to note that synthetic miRNAs are rapidly degraded after mixed with fresh plasma sample, in contrast to the endogenous miRNAs that are resistant to RNase-mediated degradation.[22] As these are artificial RNAs, they cannot be regarded as valid biological controls, but might be useful for testing the efficacy of miRNA isolation.

Our next aim was to test three reference genes, *cel-miR-39*, *hsa-miR-16* and *RNU6B* to find the most suitable candidate for normalization in our plasma samples. On the basis of the microarray analysis, all three seemed to be good for this purpose. However, by qRT-PCR *hsa-miR-16* and *cel-miR-39* were more stable candidate reference genes than *RNU6B*. In 29% of all samples, no signal for *RNU6B* was detected making this gene useless for normalization.

On the other hand, both *hsa-miR-16* and *cel-miR-39* seem to be appropriate as reference genes, however, different results have been obtained. By using *cel-miR-39* only one miRNA (*hsa-miR-181b*) has turned out to be significant, while by *hsa-miR-16* another four in total five (*hsa-miR-100, hsa-miR-181b, hsa-miR-184, hsa-miR-210* and *hsa-miR-483-5p*) have been found significantly differently expressed between ACA and ACC. Certainly, we cannot state that *hsa-miR-16* is therefore better, but in a recent study, *Chabre et al*[32] have also established *miR-483-5p* as significantly overexpressed in aggressive ACC relative to healthy individuals and ACA patients, and the underexpression of *hsa-miR-195* was also described. In our study, *hsa-miR-195* was also underexpressed in ACC plasma samples compared with ACA, but the difference has not reached statistical significance. Differences in these findings may be in part due to the different blood material used, serum and plasma (present study), respectively. High levels of circulating *hsa-miR-483-5p* and low expression of *hsa-miR-195* have been associated with shorter survival.[32] These data together may suggest that by using different reference genes either the sensitivity or the specificity can be increased. To identify the most relevant strategy for normalization, we tested various combinations of these two candidate reference genes. By using the geometric mean of *hsa-miR-16* and *cel-miR-39*, *hsa-miR-181b* and *hsa-miR-483-5p* have turned out as significantly differentially expressed in ACA compared with ACC.

To test whether the miRNAs or their combinations might be useful markers of malignancy, we have also evaluated the diagnostic potential of these significantly differently expressed miRNAs and their combinations by ROC analysis. Both the $dCT_{hsa\text{-}miR\text{-}210}$-$dCT_{hsa\text{-}miR\text{-}181b}$ and the $dCT_{hsa\text{-}miR\text{-}100}$/$dCT_{hsa\text{-}miR\text{-}181b}$ relative to hsa-miR-16 as a reference gene appeared to be the most promising markers of malignancy showing the highest diagnostic accuracy values (AUC = 0.87 and 0.85, respectively), but the sensitivity and specificity values of these markers did not seem to be high enough for its introduction in clinical practice.

On the basis of our microarray study, we have selected three potential miRNAs (*hsa-miR-192*, *hsa-mir-197* and

hsa-miR-1281) for RT-qPCR validation, but we have not found significant differences in their expression between ACA and ACC. Despite having included 100 ng total RNA in the microarray analysis, the quantity of individual miRNAs is probably very low that might be involved in the negative results of microarray analysis along with the small sample cohorts studied. Similar conclusions have been made in a study on circulating miRNAs in dogs with dilatative cardiomyopathy, where the amount of individual miRNA isolated was also very low, and the selected differentially expressed miRNAs established by microarray could not be validated by RT-qPCR.[33] In addition, Li *et al*[34] showed that only two of seven plasma miRNAs established by microarray could be validated by RT-qPCR in patients with gastric cancer. These data might suggest that the microarray technology is probably not a best choice for analyzing circulating miRNAs.

Several preanalytical and analytical factors influence the amount of miRNAs in plasma including sample processing, where the centrifugation has been shown to affect the quantity and quality of miRNA measurements.[35] We used one-step centrifugation since this is the routine laboratory practice for plasma samples and Zheng *et al* showed that two-step centrifugation resulted in loss of 10–20% of all miRNAs present in plasma. These data together suggest that standardization of sampling is required for accurate miRNA measurements.

In contrast with our microarray approach, RT-qPCR of selected miRNAs (*hsa-miR-100*,[14] *hsa-miR-181b*,[16,17] *hsa-miR-184*,[17] *hsa-miR-195*,[14,16] *hsa-miR-210*,[13,17] *hsa-miR-214*,[14] *hsa-miR-483-5p*,[13,14,16] and *hsa-miR-503*[17]) showing significant differences in studies reporting expression in tissue samples has been successful, as five of the eight selected miRNAs (*hsa-miR-100, hsa-miR-181b*, *hsa-miR-184, hsa-miR-210* and *hsa-miR-483-5p*) showed a significant overexpression in ACC plasma samples normalized to *hsa-miR-16* as a reference gene.

There are several data in the literature on the significance of these five significantly differentially expressed miRNAs in tissue tumor samples. *hsa-miR-100* was significantly downregulated in ACC compared with ACA,[14] also in childhood ACTs[36] and in primary pigmented nodular adrenocortical disease.[37] In our study on circulating miRNAs, however, *hsa-miR-100* was overexpressed in ACC samples. The expression of tissue and circulating miRNAs is not always parallel, as in the case of *hsa-miR-122* that was found to be associated with diminished expression in liver tissue but overexpressed in the serum samples of patients with hepatocellular carcinoma.[12] The molecular explanation of this discrepancy is unclear. *hsa-miR-100* regulates the insulin-like growth factor 1 receptor/mammalian target of rapamycin (IGF-1R/mTOR) pathway, which has an important role in the regulation of adrenocortical cell proliferation.[36] The upregulation of tissue *hsa-miR-181b* has been previously described in ACC compared with ACA,[16,17] as well as in several cancers[38–40] Moreover, it was associated with poor prognosis in colon adenocarcinomas.[39] *hsa-miR-181b* behaves as a tumor suppressor, and can target important oncogenes such as *B-cell lymphoma 2* (*BCL2*),[41] *TIMP metallopeptidase inhibitor 3* (*TIMP3*)[42] and *insulin-like growth factor 1 receptor* (*IGF1R*).[43] *hsa-miR-184* overexpression was noted in the squamous cell carcinoma of the tongue,[44] but its underexpression in glioma[45] was also reported. Due to the tissue-specific nature of miRNAs, the same miRNA can act as an oncogenic or as a tumor suppressor in different tissues.[46] Furthermore, in neuroblastoma cells, *hsa-miR-184* overexpression resulted in apoptosis by targeting and degradation of *serine/threonine-protein kinase* (*AKT2*), which is the main downstream effector of the potent pro-survival phosphatidylinositol 3-kinase (PI3K) pathway involved in cancer.[47] The increased activation of *AKT* has been previously described in ACC.[48] Overexpression of *hsa-miR-210* was also reported in breast cancer,[49] lung cancer[50] and pancreatic tumor[51] and several reports have shown that its expression is regulated by *hypoxia-inducible factor 1α* (*HIF1α*),[52] which is a pivotal regulatory factor overexpressed in many tumors.[50] *hsa-miR-483-5p* is one of the most investigated miRNAs in ACC. Its significant overexpression was identified in ACC compared with ACA, and was also raised as a tissue marker of malignancy.[13,14,16] *hsa-miR-483-5p* is transcribed from an intronic sequence of the *insulin-like growth factor 2* (*IGF2*) gene, that has been found to be overexpressed in ACC.[53] A significant positive correlation between overexpression of *hsa-miR-483* and *IGF2* mRNA was found in ACC tissue samples.[14]

In conclusion, our results show that there are significant differences in the expression of circulating miRNAs between ACAs and carcinomas. The microarray protocol used does not seem to be an effective method for blood-borne miRNA analysis in ACTs, whereas RT-qPCR appears to be more appropriate, as several miRNAs differentially expressed in tissues have also been confirmed to be altered in a significantly differential manner in plasma samples and some of these have also been confirmed in independent studies.[32] Standardization of sample processing including RNA extraction and the appropriate choice for reference genes seem to be the most critical steps for accurate miRNA quantification in plasma samples. Despite the good accuracy displayed, the sensitivity and specificity values of potential miRNA markers of malignancy are not high enough for clinical application at present, but further studies on larger numbers of samples might establish clinically useful markers.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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