Serum miR-483-5p and miR-195 are predictive of recurrence risk in adrenocortical cancer patients

O Chabre[1,2,3,4], R Libé[5,6,7], G Assie[5,6,7], O Barreau[5,6,7], J Bertherat[5,6,7], X Bertagna[5,6,7], J-J Feige[1,3,4] and N Cherradi[1,3,4]

[1]Institut National de la Santé et de la Recherche Médicale, Unité 1036, 17, Rue des Martyrs, F-38054 Grenoble Cedex 09, France
[2]Department of Endocrinology, University Hospital Albert Michallon, F-38043 Grenoble, France
[3]Commissariat à l'Energie Atomique, Institute of Life Sciences Research and Technologies, Biology of Cancer and Infection, 17, Rue des Martyrs, F-38054 Grenoble Cedex 09, France
[4]Lab. BCI, Univ. Grenoble Alpes, F-38041 Grenoble, France
[5]Paris Descartes University, F-75006 Paris, France
[6]Centre National de la Recherche Scientifique UMR 8104, Institut National de la Santé et de la Recherche Médicale, Unité 1016, Cochin Institute, F-75014 Paris, France
[7]Department of Endocrinology, Assistance Publique Hôpitaux de Paris, Center for Rare Adrenal Diseases, Cochin Hospital, F-75014 Paris, France

Correspondence
should be addressed
to N Cherradi
Email
nadia.cherradi@cea.fr

Abstract

Adrenocortical carcinoma (ACC) is a rare cancer with poor prognosis. Local and distant recurrences occur in a subset of tumors classified as 'aggressive' ACC (aACC), as opposed to 'non-aggressive' ACC (naACC). In this study, we investigated whether tissue and serum microRNAs (miRNAs) are predictive of ACC prognosis. Tissue miRNA expression profiles were determined using microarrays in a test series of six adrenocortical adenomas (ACAs), six naACCs, and six aACCs. Eight miRNAs were selected for further validation by quantitative RT-PCR (ten ACAs, nine naACCs, nine aACCs, and three normal adrenals). Serum levels of five miRNAs were measured in samples from 56 subjects (19 healthy controls (HC), 14 ACA, nine naACC, and 14 aACC patients). MiR-195 and miR-335 levels were significantly decreased in both tumor and serum samples of ACC patients relative to ACA patients or HC. MiR-139-5p and miR-376a levels were significantly increased in aACC compared with naACC patients in tumor samples only. Tissue miR-483-5p was markedly upregulated in a majority of ACC compared with ACA patients or HC, but most importantly, serum miR-483-5p was detected only in aACC patients. High circulating levels of miR-483-5p or low circulating levels of miR-195 were associated with both shorter recurrence-free survival ($P=0.0004$ and $P=0.0014$ respectively) and shorter overall survival ($P=0.0005$ and $P=0.0086$ respectively). In conclusion, this study reports for the first time that circulating miR-483-5p and miR-195 are promising noninvasive biomarkers with a highly specific prognostic value for the clinical outcome of ACC patients.

Introduction

Adrenocortical cancer is a rare malignancy that affects one to two people per million per year. Patients with adrenocortical carcinoma (ACC) generally have a poor prognosis, with a 5-year survival rate ranging from 15 to 30% (Allolio & Fassnacht 2006). Mitotane is the only drug currently approved for the treatment of ACC and is used for both advanced disease and adjuvant therapy (Fassnacht et al. 2012). While surgical resection may cure some patients with localized disease, most patients present with advanced disease or develop local recurrence and distant metastasis after surgery (Fassnacht et al. 2011). Benign adrenocortical tumors are much more common tumors occurring in ∼3–5% of people over 50 years old (Giordano 2006). Primary tumors of the adrenal cortex are classified by histopathological evaluation into benign and malignant groups using Weiss criteria (Weiss 1984). However, in some cases, discriminating malignant tumors from their benign counterparts remain challenging. Thus, there is a critical need for additional tools that could help the clinicians to improve their diagnosis and prognosis and to predict the therapeutic response. Gene expression profiling using DNA microarray analysis emerged as a useful technique for tumor classification (de Fraipont et al. 2005, Giordano et al. 2009, de Reynies et al. 2009, Soon et al. 2009a). Increased insulin-like growth factor 2 (IGF2) expression was identified in most studies as one of the most dominant transcriptional changes specifically present in ACC relative to adrenocortical adenoma (ACA) and normal adrenal. More recently, two-gene malignancy signature has been associated with clinical outcome of ACC patients (de Reynies et al. 2009).

MicroRNA (miRNAs) are a family of small non-coding RNA molecules that regulate gene expression at the posttranscriptional level by inducing either degradation of the mRNA transcript or translational repression (Bartel 2004). Alterations of miRNA expression contribute to tumor development, apoptosis, invasion, and metastasis, as well as to anticancer drug resistance (Esquela-Kerscher & Slack 2006). The expression patterns of tumor miRNAs can serve as molecular signatures of particular types of cancers (Calin & Croce 2006). Recent studies have reported that circulating miRNAs are readily measured in plasma or serum and that they can robustly discriminate cancer patients from healthy controls (HC), as well as distinguishing between good- and poor-prognosis patients (Lawrie et al. 2008, Brase et al. 2010). The release of miRNAs from malignant cells into the bloodstream has been attributed either to unspecific release due to tumor cell lysis or to secretion through exosomes that are shed from the cell plasma into the extracellular space then into the circulation (Brase et al. 2010).

Profiling miRNA expression in benign and malignant adrenocortical tumors led to the identification of several deregulated miRNAs in ACC compared with ACA (Soon et al. 2009b, Tombol et al. 2009, Doghman et al. 2010, Ozata et al. 2011, Patterson et al. 2011, Schmitz et al. 2011). Nevertheless, the target genes of these miRNAs as well as the functional consequences of their aberrant expressions remain to be investigated. While there is growing interest in evaluating the diagnostic potential of adrenocortical tumor miRNAs, to the best of our knowledge, no study has specifically examined the levels of circulating miRNAs or their predictive potential in adrenocortical cancer patients. In this work, we investigated on the one hand the expression profile of miRNAs in adrenocortical tumor tissues and on the other hand the absolute concentration of selected miRNAs in the serum of healthy subjects and ACA or ACC patients. We demonstrate a strong predictive value of low miR-195 and high miR-483-5p circulating levels for the diagnosis and prognosis of aggressive ACC (aACC).

Materials and methods

Patients and clinical samples

Informed signed consent for the analysis of the tumor tissue or the serum and access to the data collected were obtained from all the patients, and the study was approved by the Institutional Review Board of the Cochin Hospital. The adrenocortical tumor samples were collected between 1999 and 2011, snap frozen immediately after surgery, and kept in liquid nitrogen until use. Follow-up was performed as described previously (Abiven et al. 2006). Malignancy was assessed according to Weiss criteria (Weiss 1984) by a single experienced pathologist, and tumor staging was performed using the European Network for the Study of Adrenal Tumors classification (Fassnacht et al. 2009). ACC samples were divided into two groups: a first group consisting of aggressive tumors (recurring tumors or tumors that were already metastatic at diagnosis, aACC) and a second group consisting of non-aggressive tumors (non-recurring tumors, naACC). Recurring carcinoma was defined as a histologically verified tumor at the same localization as the surgically removed first tumor. Tumors that did not recur within

the first 3 years after surgery were considered as non-recurring carcinoma. Three normal adrenal cortex tissues (NA) were obtained from patients undergoing expanded nephrectomy for kidney cancer.

For serum samples, venous blood has been collected from patients before surgery or at time of recurrence. Postoperative serum samples were available for three ACC patients. Sera from healthy donors (ten males and nine females) were provided by a local blood transfusion center (Etablissement Français du Sang, Grenoble, France). To harvest cell-free serum, the blood was drawn into a sterile tube without anticoagulant. Samples were centrifuged at 4 °C for 10 min at 1000 g and the supernatant serum was quickly removed and stored immediately at −80 °C until analysis.

Sample RNA extraction

Isolation of total RNA from tumor samples is described in detail in Supplementary Material and Methods, see section on supplementary data given at the end of this article. For RNA isolation from serum samples, 450 µl serum was thawed on ice and lysed with an equal volume of 2× Denaturing Solution (Applied Biosystems). To allow for normalization of sample-to-sample variation in RNA isolation, synthetic non-human miRNA (Caenorhabditis elegans Cel-miR-39 50 fmol) was added to each denatured sample. RNA was isolated using the miRVana PARIS kit (Applied Biosystems) according to the manufacturer's instructions.

MiRNA profiling of adrenocortical tumors

Tumor sample labeling, hybridization of miRXplore Microarrays, and data analysis were performed as described in detail in Supplementary Material and Methods. All miRNA microarray data have been submitted to NCBI Gene Expression Omnibus database (GSE43279).

TaqMan quantitative RT-PCR assays

Tumor samples   MiRNA levels in tumor samples were measured by RT-qPCR using TaqMan miRNA assays (Applied Biosystems). Ten nanograms of tumor total RNA were reverse transcribed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Applied Biosystems) in a 15 µl RT reaction (composed of 0.15 µl 100 mM dNTPs, 1.5 µl 10× RT buffer, 0.19 µl RNase inhibitor (20 units/µl), 4.16 µl H₂O, 1 µl multiscribe reverse transcriptase, and 5 µl input RNA),

using a TGradient Thermal Cycler (Biometra, Goettingen, Germany) at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Real-time PCR was performed on the 5′-extended cDNA with Applied Biosystems TaqMan 2× Universal PCR Master Mix and the appropriate 5× TaqMan MicroRNA Assay Mix for each miRNA of interest (Assay ID: hsa-miR-195: 000494; hsa-miR-335: 000546; hsa-miR-139-5p: 002289; hsa-miR-376a: 000565; hsa-miR-376b: 001102; hsa-miR-376c: 002122; and hsa-miR-127: 000452 and RNU48: 001006). Briefly, 1.33 µl 2.5-fold diluted RT product was combined with 18.7 µl of PCR assay reagents (composed of 1 µl of TaqMan miRNA Assay, 7.67 µl of H₂O, and 10 µl TaqMan 2× Universal PCR Master Mix No Amperase UNG) to generate a PCR volume of 20 µl. Real-time PCR was carried out on C1000 Thermal cycler (CFX96 Real Time system, Bio-Rad) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed with CFX Manager Software version V1.5.534.0511 (Bio-Rad). The RNU48 RNA was used as endogenous control for normalization. Normalized expression was calculated using the comparative CT method and fold changes were derived from the $2^{-\Delta\Delta Ct}$ values for each miRNA.

Serum samples   For measurement of circulating miR-NAs, as the yield of RNA from small volume of serum samples (i.e. 450 µl) is below the limit of accurate quantitation by spectrophotometry, we chose to use a fixed volume of RNA eluate from a given volume of starting serum rather than a fixed mass of input RNA into the RT reaction (Mitchell et al. 2008). RNA solution (2.5 µl) from the ∼45 µl eluate from RNA isolation of a given sample was used as input into the RT reaction (composed of 0.15 µl 100 mM dNTPs, 1.5 µl 10× RT buffer, 0.19 µl RNase-Inhibitor (20 units/µl), 6.66 µl H₂O, 1 µl multiscribe reverse transcriptase, and 2.5 µl input RNA). For samples in which RNA was isolated from 450 µl serum, for example, 2.5 µl eluate represents the RNA corresponding to (2.5/45)×450=25 µl of serum. Real-time PCR was performed as described earlier for RNA samples from tumor tissue, with slight modifications: 4.5 µl of sevenfold diluted RT product was combined with 5.5 µl PCR assay reagents (composed of 0.5 µl TaqMan miRNA Assay and 5 µl TaqMan 2× Universal PCR Master Mix, No Amperase UNG, Applied Biosystems) to generate a PCR volume of 10 µl (Assay ID: hsa-miR-195: 000494; hsa-miR-335: 000546; hsa-miR-139-5p: 002289; hsa-miR-376a: 000565; hsa-miR-483-5p: 002338; and Cel-miR-39: 000200). Standard curves were generated for absolute quantification of each miRNA. A dilution series of each

synthetic RNA oligonucleotides corresponding to mature miRNA was made in water such that the final input into RT reaction had a final volume of 2.5 μl. A line was fitted from each dilution series using cycle threshold (Ct) values within the linear range, from which $y = ax + b$ equations were derived for quantification of absolute miRNA copies (x) from each sample Ct (y). Absolute copies of miRNA input into the RT reaction were converted to copies per milliliter of serum based on the knowledge that the material input corresponds to RNA from 5.5% of the total starting volume of plasma (2.5 μl of the total RNA eluate volume (45 μl on average) was input into the RT reaction).

Normalization of serum miRNA qRT-PCR data using spiked-in C. elegans miR-39

As no established circulating small RNA control exists for normalization of technical variations in serum sample processing, data normalization was based upon spiked-in Cel-miR-39 (Mitchell *et al.* 2008). The median of the spiked-in Cel-miR-39 Ct values obtained from all the samples to be compared was calculated. A normalization factor was then calculated for each sample based on the formula:

Normalization factor

$$= \frac{1}{[2^{(\text{Median \_ SpikeIn } Ct \text{ value}) - (\text{SpikeIn } Ct \text{ value of the given sample})}]}$$

The number of copies of a given miRNA in each sample was multiplied by the normalization factor corresponding to the sample to obtain a normalized copy number value.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software version 4.03 (San Diego, CA, USA). Kruskal–Wallis test was performed to compare the different levels of miRNA expression. Results are expressed as means ±S.E.M. The ability of miRNA to provide diagnostic and prognostic information for patients with malignant disease was evaluated using follow-up data. Receiver operating characteristic (ROC) curves were performed using MedCalc Software version 12.3.0.0 (Mariakerke, Belgium). Correlation analysis was carried out using two-tailed Spearman correlation test. Survival analysis was performed using the Kaplan–Meier method and curves were compared using the log-rank test. High and low expressions for miR-195 and miR-483-5p were defined with reference to the expression cutoff value determined by ROC analyses. A P value of 0.05 was considered as statistically significant.

Results

Patient characteristics

We performed miRNA microarray analysis on RNA samples from a first panel of 18 adrenocortical tumors (test cohort: six adenomas (ACA), six aACC, and six naACC). Results were subsequently validated on samples from a second panel of 28 patients (validation cohort: ten ACA, nine aACC, and nine naACC). Table 1 summarizes their clinical characteristics. aACC patients from both cohorts had stage II (n=4), stage III (n=2), or stage IV (n=9) tumors according to ENS@T classification (Fassnacht *et al.* 2009). Among the aACC patients having stage II tumors, three of them died of disease recurrence (median overall survival of 34 months, range 27.6–85.3 months) and one patient was alive at the time of the last follow-up. Most of the patients with stage III or stage IV tumors died of metastatic lesions with a median overall survival of 11.4 months (range 0.5–74.4 months) except one patient with stage IV tumor who died from post-operative complications (patient 18). naACC patients (n=15) had stage I (n=3) or stage II (n=12) tumors. All of them were alive at the time of the last follow-up (mean follow-up 6.8 years, range 3.5–12.6 years, Table 1).

Serum miRNA analysis was performed on a third panel of 37 patients (Table 2) including 14 ACA, 14 aACC, and 9 naACC patients and 19 healthy donors. aACC patients had recurring stage II (n=6) or stage III (n=2) tumors or metastatic at diagnosis stage IV tumors (n=6). In the eight stage II and stage III patients, the median recurrence-free survival was 8.3 months (range 4–36 months). Five of them died of disease recurrence. In the six stage IV patients, five died of their disease (median overall survival 3.5 months, range 0.9–45.3 months), and one patient was alive with metastasis at the time of the last follow-up (patient 43). naACC patients (n=9) had stage I (n=3) or stage II (n=6) tumors. They were all alive at the time of the last follow-up (mean follow-up 4.1 years, range 0.7–10.1 years, Table 2) except one patient who died from an unrelated cause (patient 53). As the follow-up of patients 52 and 53 was <3 years (0.8 and 0.7 years respectively), they were not used for comparison between aACC and naACC but were used for the comparison between ACA and ACC samples.

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Endocrine-Related Cancer

Table 1 Clinical characteristics of the patients (tumor tissue cohorts).

Patient ID	Age	Sex	Tumor size (mm)	Weiss score	ENS@T stage	ACC subgroup	Hormonal status	Recurrence (Y/N)	RFS (months)	Metastasis at diagnosis	Status	Survival (months)	Follow-up (years)
Test cohort													
1	41	F	140	8	II	a	GC/AD/Precs	Y	19	N	Dead	34	2.9
2	45	F	130	9	III	a	GC/AD	–	–	N	Dead	12.4	1.2
3	67	F	60	6	IV	a	GC/AD	–	–	Y	Dead	22.2	2.5
4	24	F	78	9	IV	a	GC/AD	–	–	Y	Dead	9.4	0.9
5	68	F	110	9	IV	a	GC/AD	–	–	Y	Dead	2.4	1.1
6	43	F	80	8	IV	a	AD	–	–	Y	Dead	45.3	5.2
7	49	F	110	5	II	na	AD/ES	N	–	N	Alive	–	3.4
8	31	M	82	3	II	na	NS	N	–	N	Alive	–	5.8
9	63	M	82	5	II	na	NS	N	–	N	Alive	–	5.9
10	31	F	83	6	II	na	NS	N	–	N	Alive	–	5.1
11	40	F	85	6	II	na	NS	N	–	N	Alive	–	3.9
12	45	F	98	5	II	na	NS	N	–	N	Alive	–	4.5
Validation cohort													
13	59	F	160	6	II	a	GC/AD	Y	10.9	N	Alive	–	2.7
14	39	M	110	3	II	a	GC	Y	24	N	Dead	27.6	2.3
15	51	F	100	7	II	a	GC/AD	Y	22	N	Dead	85.3	7.6
16	43	F	80	6	III	a	GC/AD	Y	6.2	N	Dead	8.6	0.9
17	46	F	110	8	IV	a	GC/AD	–	–	Y	Dead	11.4	1.1
18	63	M	80	5	IV	a	GC	–	–	Y	Dead	1[a]	0.1
19	47	F	240	9	IV	a	GC/AD	–	–	Y	Dead	11.3	1.2
20	73	M	120	8	IV	a	GC/ES	–	–	Y	Dead	0.5	0.3
21	29	M	160	8	IV	a	GC/AD	–	–	Y	Dead	74.4	6.1
22	16	F	45	2	I	na	GC/AD	N	–	N	Alive	–	11.1
23	25	F	50	3	I	na	GC/AD	N	–	N	Alive	–	11
24	41	F	50	3	I	na	GC	N	–	N	Alive	–	6.4
25	26	F	130	7	II	na	AD	N	–	N	Alive	–	6.2
26	53	F	70	4	II	na	GC/AD	N	–	N	Alive	–	4.3
27	42	F	55	4	II	na	GC/AD	N	–	N	Alive	–	3.5
28	54	F	100	6	II	na	NS	N	–	N	Alive	–	10.9
29	30	M	250	5	II	na	NS	N	–	N	Alive	–	7.2
30	70	F	55	4	II	na	NS	N	–	N	Alive	–	12.6

F, female; M, male; a, aggressive ACC group; na, non-aggressive ACC group; GC, glucocorticoids; AD, androgens; ES, estrogens; Precs, precursors; NS, non-secreting; Y, yes; N, no; RFS, recurrence-free survival.
[a]Patient 18 died from postoperative complications.

Endocrine-Related Cancer

Table 2 Clinical characteristics of the patients (sera cohort).

Patient ID	Age	Sex	Tumor size (mm)	Weiss score	ENS@T stage	ACC subgroup	Hormonal status	Recurrence (Y/N)	RFS (months)	Metastasis at diagnosis	Status	Survival (months)	Follow-up (years)
Sera cohort													
31	75	F	90	7	II	a	GC/AD	Y	6.4	N	Dead	51.6	4.6
32	61	F	135	7	II	a	AD	Y	5.5	N	Alive	–	5.3
33	20	M	130	5	II	a	NS	Y	36	N	Alive	–	7.7
34	72	F	100	5	II	a	NS	Y	11.6	N	Dead	15.5	1.5
35	53	F	110	9	II	a	GC	Y	4	N	Dead	6.3	0.9
36	68	M	70	6	II	a	NS	Y	10.2	N	Dead	67	5.7
37	30	F	250	6	III	a	AD	Y	16.7	N	Alive	–	4.2
38	63	M	100	6	III	a	NS	Y	5.2	N	Dead	16.7	1.6
39	43	F	80	8	IV	a	GC	–	–	Y	Dead	45.3	5.2
40	67	F	60	5	IV	a	GC/AD	–	–	Y	Dead	22.2	2.5
41	33	M	190	9	IV	a	Subclinic CS	–	–	Y	Dead	0.9	0.3
42	55	F	52	5	IV	a	GC/AD	–	–	Y	Dead	3.5	0.5
43	55	M	150	9	IV	a	GC	–	–	Y	Alive	–	1.8
44	62	F	43	8	IV	a	GC/AD	–	–	Y	Dead	2.2	0.4
45	34	F	32	7	I	na	Subclinic CS	N		N	Alive	–	4.3
46	29	F	40	3	I	na	GC	N		N	Alive	–	3.6
47	50	F	22	4	I	na	NS	N		N	Alive	–	4.5
48	71	F	70	6	II	na	NS	N		N	Alive	–	10.1
49	31	F	83	6	II	na	NS	N		N	Alive	–	5.1
50	37	F	85	5	II	na	Subclinic CS	N		N	Alive	–	4.2
51	55	F	62	7	II	na	NS	N		N	Alive	–	3.8
52	53	F	45	3	II	na	GC/AD	N		N	Alive	–	0.8[a]
53	67	F	44	6	II	na	GC/AD/Precs	N		N	Dead	6.1[b]	0.7[a]

F, female; M, male; a, aggressive ACC group; na, non-aggressive ACC group; GC, glucocorticoids; AD, androgens; ES, estrogens; Precs, precursors; Subclinical CS, subclinical Cushing's syndrome; NS, non-secreting; Y, yes; N, no; RFS, recurrence-free survival.

[a]Patients 52 and 53 were excluded from the naACC group when compared with the aACC group as elapsed time since surgery was too short. They were however included in the AAC group when compared with the ACA group.

[b]Patient 53 died from unrelated cause.

Tissue miRNA expression in ACA and ACC patients

We used a microarray-based approach to measure the expression of tissue miRNAs in a test cohort of ACA and ACC. SAM analysis was performed to identify the most significantly deregulated miRNAs that could distinguish adenomas from carcinomas (combined aACC and naACC) and distinguish aACC from naACC. Twelve miRNAs were found to be significantly differentially expressed between ACA and ACC (Supplementary Figure 1, see section on supplementary data given at the end of this article). Five miRNAs (miR-335, miR-195, miR-497, mir-199A-3P, and miR-199A-5P) were underexpressed whereas seven miRNAs (miR-503, miR-514, miR-509-3P, miR-93, miR-148B, miR-508-3P, and miR-513A-5P) were overexpressed in ACC compared with those in ACA. MiR-335 and miR-195 appeared to be the best discriminatory miRNA between ACA and ACC ($P=0.0063$ and $P=0.0010$ for miR-335 and miR-195 respectively). Twenty-nine miRNAs were significantly differentially expressed between naACC and ACC (Supplementary Figure 2). All discriminatory miRNAs were overexpressed in aACC compared with those in naACC. Among them, miR-127, miR-376a, miR-376b, miR-376c, and miR-130a are slightly more activated in aACC while strongly repressed in naACC. MiR-495 is only slightly repressed in aACC while strongly repressed in naACC samples. MiR-139-5p allowed to discriminate between the two ACC groups as it showed a significant overexpression in aACC compared with naACC ($P=0.039$).

We selected miRNAs for RT-qPCR validation in the test cohort and in an independent cohort of ten ACA, nine naACC, nine aACC, and three normal adrenal cortices (NA). Because miR-483-5p, a miRNA located within the second intron of *IGF2* gene, was reported to be over-expressed in aACC compared with ACA (Soon *et al.* 2009*b*, Ozata *et al.* 2011, Patterson *et al.* 2011), it was also included in our analyses. The levels of miR-195, miR-335, miR-139-5p, miR-127, miR-376a, miR-376b, miR-376c and miR-483-5p were not significantly different between ACA and NA (Supplementary Table 1, see section on supplementary data given at the end of this article). We confirmed a significantly lower expression of miR-195 and miR-335 in ACC compared with ACA and a significant overexpression of miR-139-5p, miR-376a, miR-376b, and miR-376c in aACC compared with naACC (Fig. 1 and Supplementary Figure 3). MiR-483-5p was markedly upregulated in both naACC (6/9) and aACC (8/9) of the validation cohort compared with ACA (Fig. 1). However, miR-483-5p expression levels were not statistically different between aACC and naACC samples (mean expressions: 19.5 ± 4.9

and 34.0 ± 11.4 for naACC and aACC respectively, $P=0.24$, Fig. 1). Interestingly, the human ACC cell line NCI H295R (Bird *et al.* 1993) and aACC samples displayed similar expression patterns of all selected miRNA (data not shown). Remarkably, 14 of the 29 miRNAs upregulated in aACC compared with naACC were located in the 14q32 locus (Supplementary Table 2).

MiRNA-gene target pairs in ACC

To identify putative gene targets of the differentially expressed miRNAs detected in this study, we selected only the miRNA-gene targets that were predicted by at least three different bioinformatic algorithms (miRWalk: http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/); miRanda: http://www.microrna.org/; TargetScan: http://www.targetscan.org/). We then integrated our miRNA data and previously published gene expression analysis data (de Reynies *et al.* 2009) and selected only the miRNA-gene target pairs that were expressed in patients with ACC and were inversely correlated. We identified nine miRNA-target pairs that are potentially involved in ACC pathogenesis (Supplementary Table 3, see section on supplementary data given at the end of this article). Most of the discriminatory miRNA targets appear to be involved in Wnt pathway, cell proliferation, and cell cycle progression.

Diagnostic potential of selected tumor miRNAs

We next determined the diagnostic value of tissue miR-195, miR-335, miR-483-5p, miR-139-5p, and miR-376a. ROC analyses indicated high diagnostic accuracy for miR-195, miR-335, and miR-483-5p in discriminating benign (ACA) from malignant (ACC) tumors (area under the curve (AUC) of 0.83 for miR-195, 95% CI: 0.69–0.92, $P<0.0001$; AUC of 0.87 for miR-335, 95% CI: 0.75–0.95, $P<0.0001$; AUC of 0.90 for miR-483-5p, 95% CI: 0.74–0.98, $P<0.0001$, Fig. 2A). MiR-139-5p and miR-376a were the most informative miRNAs for the discrimination between aACC and naACC subgroups (Fig. 2B, AUC of 0.94 for miR-139-5p, 95% CI: 0.79–0.99, $P<0.0001$; AUC of 0.89 for miR-376a, 95% CI 0.68–0.98, $P<0.0001$).

Serum levels of five miRNAs in ACA and ACC patients

Given that the levels of miR-195, miR-335, miR-483-5p, miR-139-5p and miR-376a were significantly deregulated in ACC tumor tissues, we proceeded to screen for expression of these five miRNAs in serum samples from ACA ($n=14$) and ACC patients ($n=23$, Table 2) as well as
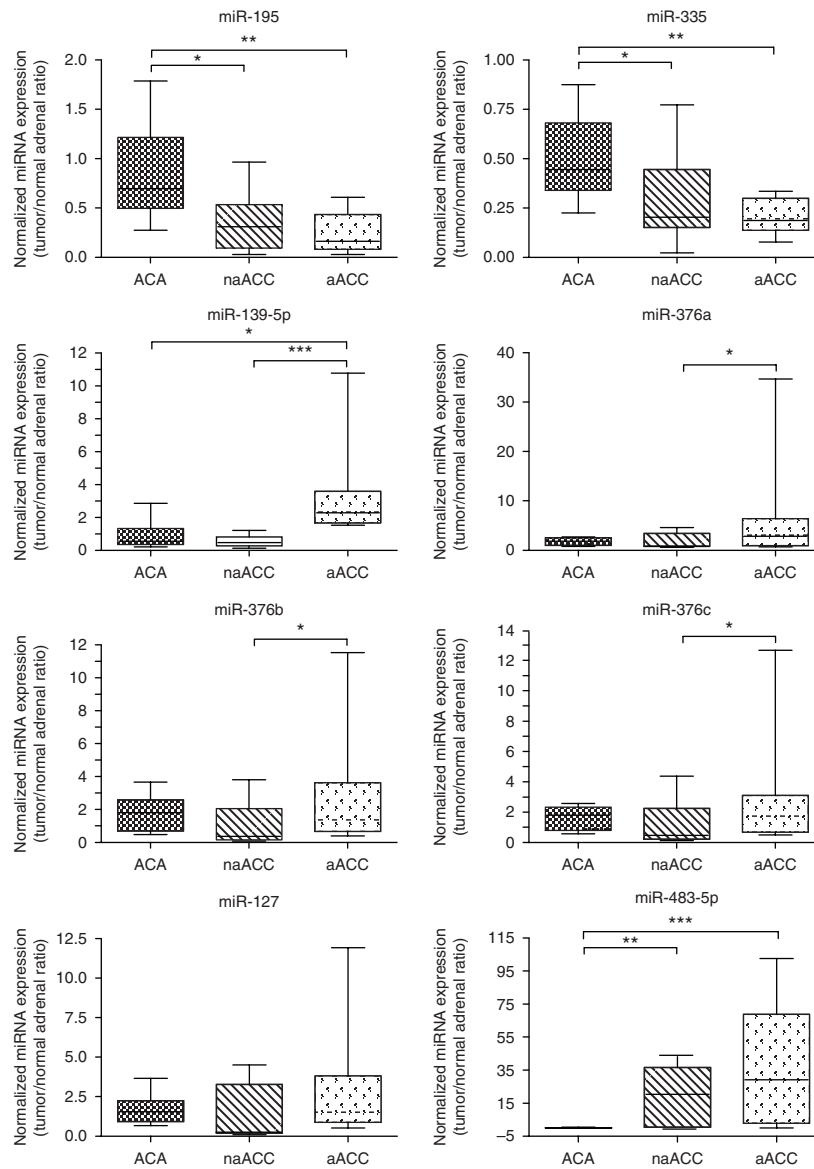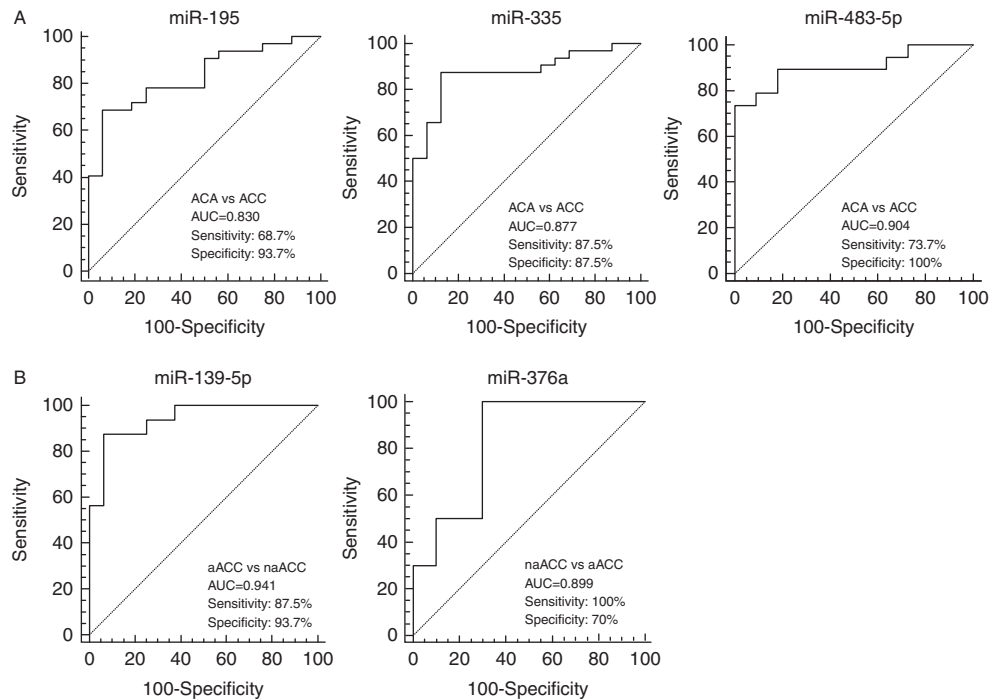
Figure 1

TaqMan quantitative RT-PCR analysis of selected miRNA in ACA (adenomas), non-aggressive carcinoma (naACC), and aggressive carcinoma (aACC) samples of the validation cohort. Expression values of each miRNA were normalized to RNU48. Box plots represent miRNA expression levels in tumor samples as a ratio to miRNA expression levels in normal adrenocortical tissue samples. Although there was a trend toward an increase in miR-127 expression in the aACC group compared with the naACC group, this was not statistically significant. The lines inside the boxes denote the medians. Statistical analysis was performed using Kruskal–Wallis test for unpaired data and $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

from 19 HC. We first evaluated the intra- and interassay variability for circulating miRNA measurements. Due to significant sample-to-sample variability in both protein and lipid content of serum samples, which could affect efficiency of RNA extraction, exogenous Cel-miR-39 was spiked-in after the initial denaturation, providing an internal reference for normalization (Mitchell et al. 2008). Four independent RNA extractions were performed using the same pool of three normal sera. Cel-miR-39 and miR-139-5p were repeatedly amplified three times daily for 4 continuous days. The intra-assay coefficient of variation (CV) ranged from 2.3 to 7.5% whereas the interassay CV ranged from 5.0 to 8.8%. Comparison of the four RNA extractions yielded a higher CV (9.5%), suggesting that most of the variability is linked to the RNA extraction process.

Figure 2

Diagnostic value of tissue miR-195, miR-335, miR-483-5p, miR-376a, and miR-139-5p. (A) ROC curve analyses of tumor miR-195, miR-335, and miR-483-5p for discrimination of patients with ACA from patients with ACC (combined aACC and naACC samples). (B) ROC curve analyses of tumor miR-376a and miR-139-5p for discrimination of patients with aACC from patients with naACC.

Quantification of serum levels of miR-195, miR-335, miR-483-5p, miR-139-5p, and miR-376a yielded different ranges of threshold cycles (Supplementary Table 4, see section on supplementary data given at the end of this article). The mean recovery of spiked-in Cel-miR-39 was $47.8 \pm 15.4\%$ in HC, $32.1 \pm 8.1\%$ in ACA patients, $41.5 \pm 8.7\%$ in naACC patients, and $29.9 \pm 7.4\%$ in aACC patients. Statistical analysis revealed that extraction efficiency was significantly different between certain serum sample groups, indicating that normalization to an internal calibrator is essential (HC vs ACA, $P < 0.01$; HC vs naACC: $P > 0.05$; HC vs aACC, $P < 0.001$; ACA vs naACC, $P > 0.05$; ACA vs aACC, $P > 0.05$; and naACC vs aACC $P < 0.05$). As shown in Fig. 3, no difference was observed in the levels of circulating miR-195 and miR-335 between HC and ACA patients. By contrast, we found that miR-195 and miR-335 levels were significantly decreased in the serum of ACC patients (combined aACC and naACC) when compared with the serum of HC or ACA patients (miR-195: $P < 0.001$ for HC vs ACC and $P < 0.001$ for ACA vs ACC; miR-335: $P < 0.05$ for HC vs ACC and $P < 0.01$ for ACA vs ACC). ACC serum samples displayed a significant increase in the levels of miR-139-5p when compared with

HC or to ACA samples ($P < 0.05$ for HC vs ACC and $P < 0.05$ for ACA vs ACC, Fig. 3). However, no difference was found in the levels of miR-139-5p between aACC and naACC serum samples ($P = 0.1478$), suggesting a lack of correlation between tissue and serum expression of miR-139-5p. In addition, miR-376a circulating levels were significantly decreased in ACC patients compared with HC or to ACA patients (Fig. 3), suggesting an inverse correlation between miR-376a tumor content and circulating miR-376a in ACC patients. As we were provided with matched tissue-serum samples from a stage IV ACC patient (patient 6 of the test cohort corresponding to patient 39 of the sera cohort), we then compared miR-376a levels in both samples. Interestingly, the tumor of this patient displayed a marked overexpression of miR-376a by fivefold compared with normal tissue (Supplementary Figure 3) whereas the circulating levels of miR-376a in this patient (10 400 copies/ml) were far below the mean-copy number in HC (164 000 ± 28 000 copies/ml). MiR-483-5p was detected neither in HC nor in ACA or naACC patients (Fig. 3). By contrast, sera from aACC patients displayed high levels of miR-483-5p. Thus, circulating miR-483-5P appears to be highly specific of aggressive tumors.

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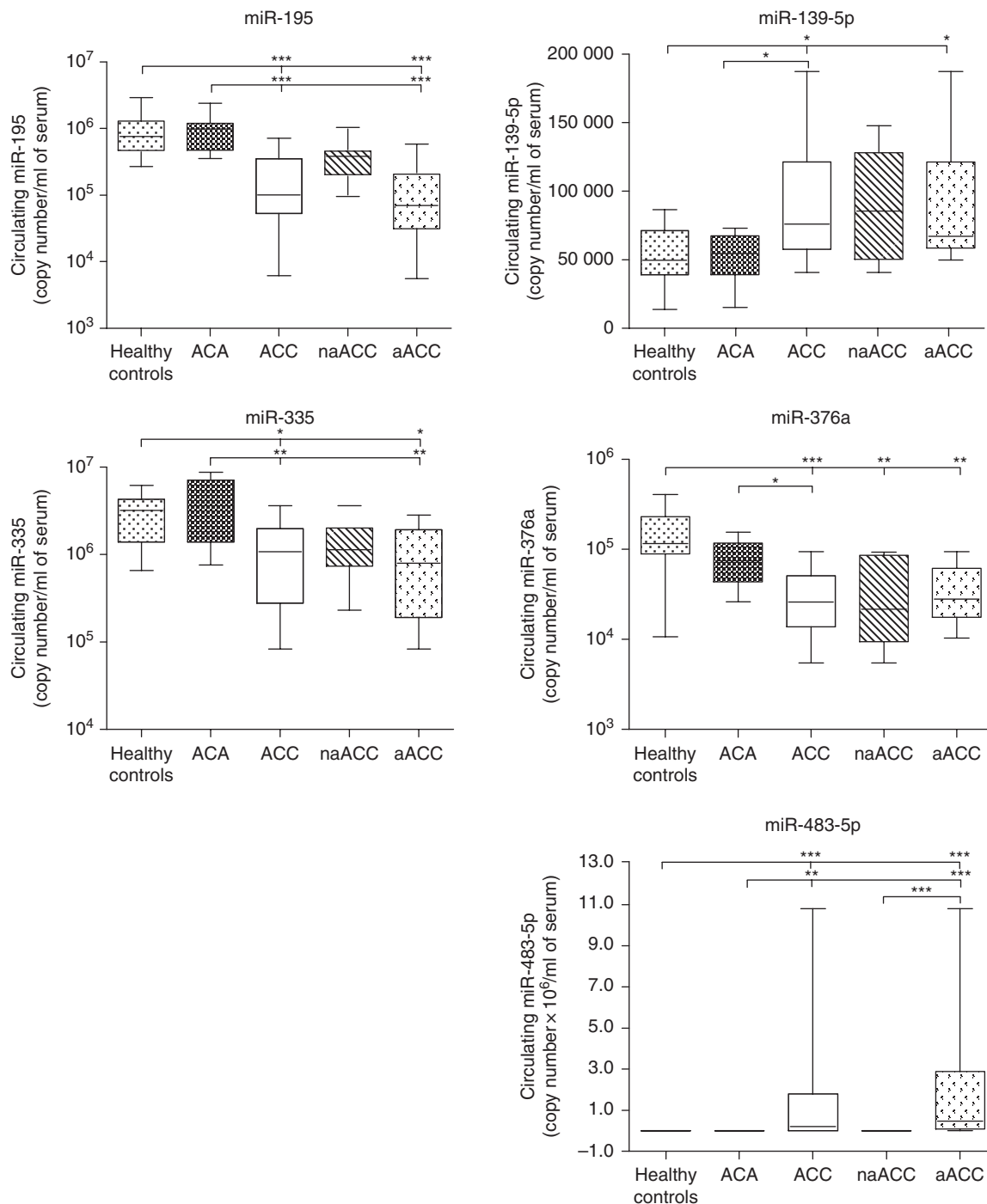
Figure 3

Serum levels of miR-195, miR-335, miR-139-5p, miR-376a, and miR-483-5p in healthy controls ($n=19$), ACA ($n=14$), and ACC ($n=23$, 9 naACC + 14 aACC) patients. MiRNA levels in naACC and aACC patients were also plotted separately. Ct values were converted to absolute number of copies/ml using a dilution series of known input quantity of synthetic target miRNA run simultaneously as the experimental samples. Mean values for circulating miR-195 were $(1.03 \pm 0.16) \times 10^6$ copies/ml in healthy controls $(0.89 \pm 0.15) \times 10^6$ copies/ml in ACA patients and $(0.22 \pm 0.05) \times 10^6$ copies/ml in ACC

patients. Serum miR-483-5p levels were below the detection limit in healthy controls and ACA and naACC patients (>35 Ct). The median value for miR-483-5p in aACC patients was 472 000 copies/ml (range 170 000–10^7). Of note, 2/15 patients of the aACC group had no detectable circulating miR-483-5p (patients 31 and 37 with stage II and stage III ACC respectively). Statistical analysis was performed using Kruskal–Wallis test for unpaired data and $P<0.05$ was considered significant (*$P<0.05$, **$P<0.01$, and ***$P<0.001$).

Diagnostic values of circulating miRNAs for ACA and ACC patients

Based on the identification of deregulated levels of miR-195, miR-335, miR-139-5p, miR-376a, and miR-483-5p in ACC patients, we next assessed their potential use as diagnostic markers. ROC curves were plotted for each miRNA based on RT-qPCR values (Fig. 4). The most informative miRNA for the discrimination of ACA from ACC patients was miR-195 (AUC=0.948, 95% CI: 0.819–0.994, $P<0.0001$). MiR-195 could detect individuals with adrenocortical cancer with 90.9% sensitivity and 100% specificity. MiR-335 and miR-376a were also good markers of malignancy with an AUC of 0.837, and 0.811 respectively (Fig. 4). Although miR-139-5p displayed high sensitivity for the discrimination of ACA from ACC patients (87.5%), its specificity was moderate (65%, AUC= 0.714, $P=0.023$, Fig. 4). Importantly, miR-483-5P could distinguish naACC from aACC patients with 85.7% sensitivity and 100% specificity (AUC=0.929, 95% CI: 0.741–0.994, $P<0.0001$, Fig. 4).

Correlation between hormonal status and miRNA levels

To investigate a possible association between miRNA expression patterns and steroid hormone secretion, we studied the correlation of urinary free cortisol (UFC), 17-OH steroids, and DHEA-S levels (Supplementary Tables 5 and 6, see section on supplementary data given at the end of this article) with tissue or serum miRNA signatures in ACC patients. We did not find any correlation between tissue or serum levels of miR-195, miR-335, miR-376A, or miR-483-5p and UFC, DHEA-S, or 17-OH progesterone levels (not shown). By contrast, a positive correlation was observed between miR-139-5p expression and UFC levels in both tissue ($r=0.532$, $P=0.034$) and serum samples ($r=0.604$, $P=0.022$, Supplementary Figure 4).

Relationship between circulating miR-195 and miR-483-5p and tumor stage or tumor size

Given that miR-195 and miR-483-5p had the greatest diagnostic accuracy, we searched for possible associations between serum miR-195 or miR-483-5p levels and tumor stage or tumor size. As shown in Fig. 5A, miR-195 circulating levels were significantly lower in stage II aACC patients compared with stage II naACC patients ($P=0.030$). By contrast, no significant difference in circulating miR-195 was observed between stage II, stage III, and stage IV aACC patients. Serum levels of miR-483-

5p were significantly higher in stage II aACC compared with stage II naACC patients (Fig. 5A, $P=0.006$) as well as in stage IV aACC compared with stage II aACC patients ($P=0.042$). We found that low circulating levels of miR-195 tended to be inversely correlated with tumor size (Fig. 5B, $r=0.397$, $P=0.06$) whereas high circulating levels of mir-483-5p were positively correlated with tumor size ($r=0.551$, $P=0.005$).

Survival and recurrence predictive value of serum miR-195 and miR-483-5p

Of the 23 ACC patients whose circulating miRNAs were analyzed by RT-qPCR, one had insufficient follow-up and one had died from unrelated cause (patients 52 and 53, Table 2). We could however include 21 patients in a retrospective study of overall survival. The median duration of follow-up was 25.8 months (range 3.6–92.4 months) for the aACC group and 51.6 months (range 43.2–121.2 months) for the naACC group (Table 2). Kaplan–Meier analysis according to ROC-derived cutoff value of miR-195 revealed that the group with low circulating miR-195 had significantly worse overall survival (22.2 months, $n=9$; hazard ratio (HR): 6.10; 95% CI: 1.54–19.8, log-rank test $P=0.0086$) compared with the high miR-195 group ($n=12$) in which the only two patients who died had metastatic lesions at diagnosis (Fig. 6A). Patients with high circulating levels of miR-483-5p expression had also a poorer survival ($n=11$, 16.7 months, HR: 0.066; 95% CI: 0.033–0.183, Log-rank test $P=0.0005$) compared with the low miR-483-5p group ($n=10$, Fig. 6A). Although our cohort was relatively small, we attempted to analyze recurrence-free survival as a function of miR-195 and miR-483-5p circulating levels in each patient. The six patients with tumors that were already metastatic before surgery were excluded from the analysis. As shown in Fig. 6B, patients with low miR-195 had a median recurrence-free survival of 8.3 months (HR: 8.0; 95% CI: 2.78–72.8, Log-rank test $P=0.0014$) whereas patients with high miR-483-5p levels had a median recurrence-free survival of 5.5 months (HR: 0.13; 95% CI: 0.004–0.215, Log-rank test $P=0.0004$). These results indicate that both miRNAs may serve as molecular biomarkers of high risk of recurrence for ACC patients.

Expression of miR-195 and miR-483-5p in postoperative serum samples

Considering the potential of circulating miR-195 and miR-483-5p as biomarkers for ACC, we measured the levels of

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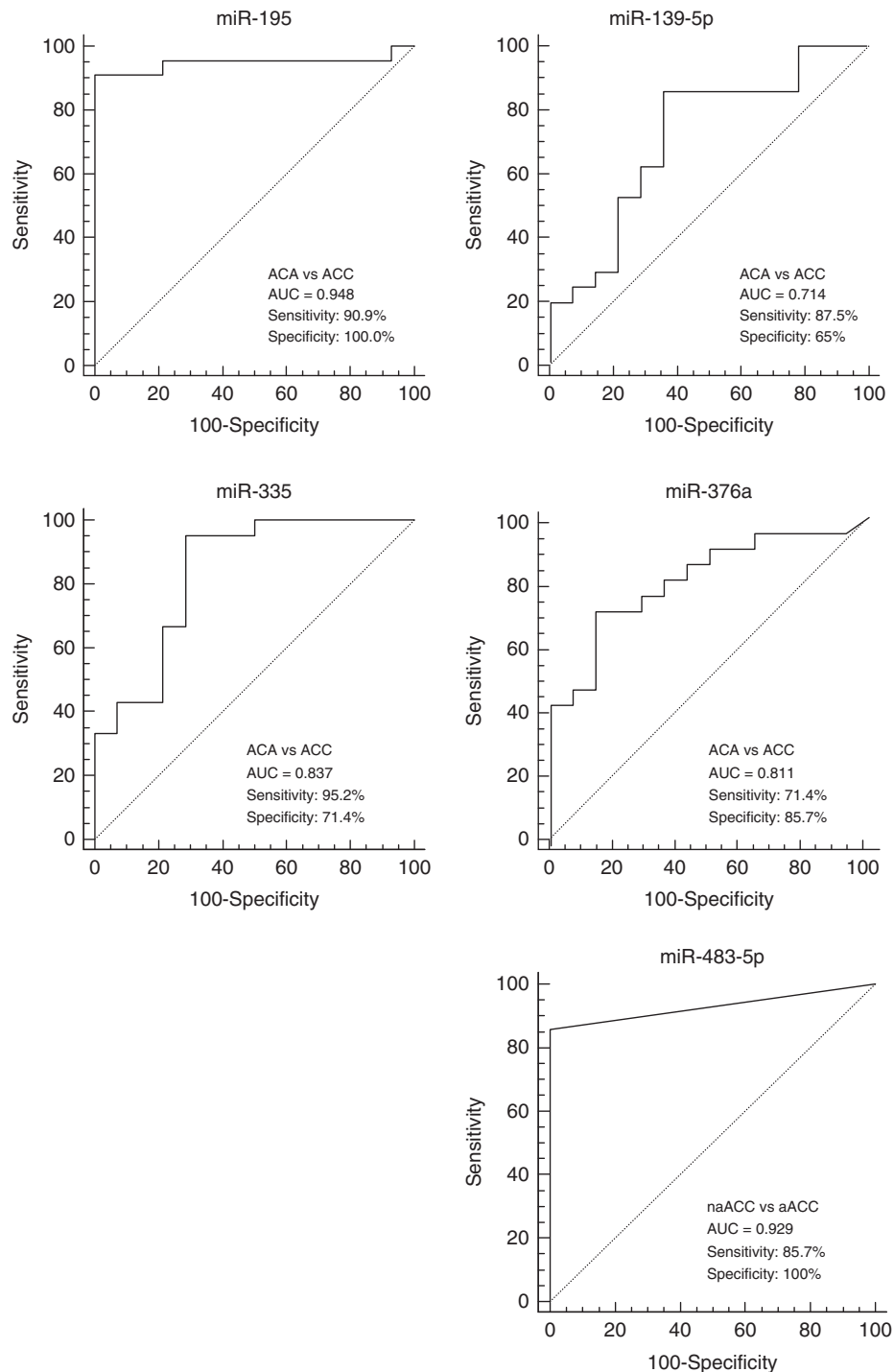
Figure 4

Diagnostic values of circulating miR-195, miR-335, miR-139-5p, miR-376a, and miR-483-5p in discriminating ACA from ACC patients (combined aACC and naACC samples) or naACC from aACC patients. ROC curves were plotted for each miRNA based on RT-qPCR values.

these miRNAs in postoperative sera that were available from three aACC and naACC patients (Supplementary Figure 5, see section on supplementary data given at the end of this article). MiR-195 was increased after surgery in both patients 43 and 49 by 2.75- and 4-fold respectively to reach levels comparable with those measured in HC. Only a slight increase in miR-195 was observed in patient 32 after tumor resection. MiR-483-5p was still undetectable in

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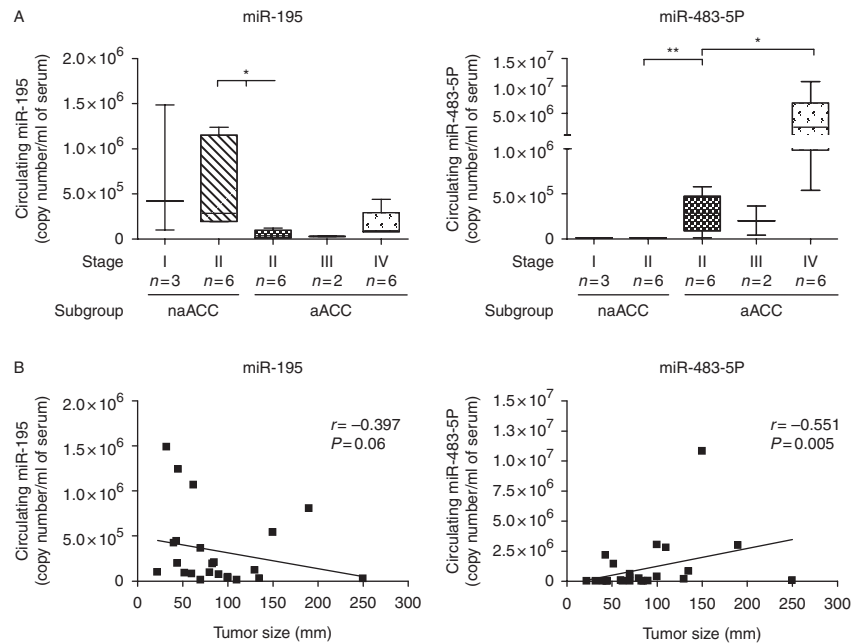
Figure 5

Circulating miR-195 and miR-483-5p levels according to the ACC tumor stage (A) or the tumor size (B). Correlation between miR-195 or miR-483-5p and tumor size was analyzed using Spearman's test. Statistical analysis was performed using the Kruskal–Wallis test, $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$).

patient 49 (from the naACC group) whereas it was markedly decreased in patients 32 and 43 after tumor resection. Altogether, these findings suggest a link between the deregulation of circulating miR-195 and miR-483-5p and the tumor of these patients.

Discussion

The development of noninvasive reliable biomarkers represents a major clinical challenge for the management of patients with adrenocortical cancer. Previous miRNA expression profiling of benign and malignant adrenocortical tumors provided potential diagnostic and prognostic tools for the disease (Soon et al. 2009b, Tombol et al. 2009, Ozata et al. 2011, Patterson et al. 2011, Schmitz et al. 2011). To further strengthen these observations, we first analyzed miRNA expression in ACA and ACC tissues and subsequently investigated the circulating levels of differentially expressed miRNAs in patient sera. One major interest of our work is the use of two types of ACCs (aACC and naACCs) that were associated with different clinico-pathological features (stage, recurrence and/or metastasis) and different outcomes. We present here the first study demonstrating the potential diagnostic and prognostic value of serum miRNAs in adrenocortical cancer.

Specifically, we provide evidence that serum miR-483-5p and miR-195 are promising noninvasive biomarkers for improved identification of high-risk ACC patients.

In agreement with previous reports (Soon et al. 2009b, Doghman et al. 2010, Ozata et al. 2011, Patterson et al. 2011, Schmitz et al. 2011), our RT-qPCR analyses revealed that miR-195 and miR-335 were downregulated in ACC ($n = 30$) compared with ACA ($n = 16$) tumor tissues. A trend toward an increase in miR-483-5p in ACC tissues compared with ACA was first reported by Soon et al. (2009b), but this difference did not reach statistical significance. Similarly, microarray analysis of our test cohort indicated that miR-483-5p expression was not significantly different between ACC and ACA ($P = 0.08$, data not shown). However, in line with more recent studies (Ozata et al. 2011, Patterson et al. 2011), our RT-qPCR analyses revealed a marked upregulation of miR-483-5p in ACC compared with ACA of the validation cohort. Among the miRNAs differentially expressed between aACC and naACC, miR-503, miR-210, miR-542-5p, miR-320a, miR-93, and miR-148b have been reported previously to be upregulated in ACC compared with ACA (Soon et al. 2009b, Tombol et al. 2009, Ozata et al. 2011, Schmitz et al. 2011). More importantly, we identified miR-139-5p and miR-376a as new potent diagnostic
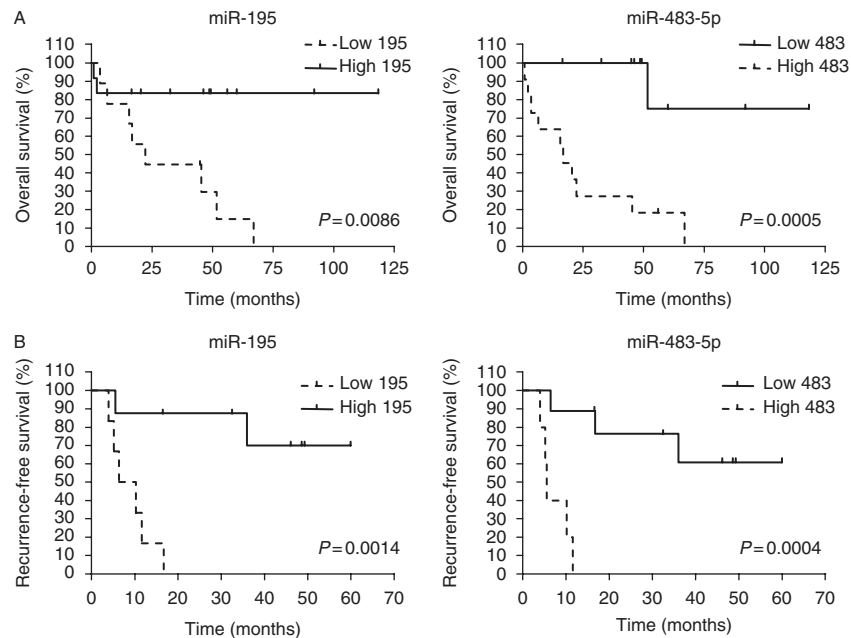
Figure 6

Kaplan–Meier survival analysis according to miR-195 and miR-483-5p circulating levels in 21 ACC patients. (A) Overall survival. Low levels of miR-195 or high levels of miR-483-5p are associated with poor survival. (B) Recurrence-free survival as a function of miR-195 and miR-483-5p circulating levels. Low level of miR-195 corresponds to a value lower than the cutoff derived from ROC curve analyses. For miR-483-5p, low level refers to undetectable level using RT-qPCR. P values were determined using a log-rank test.

markers that appear to accurately discriminate between non-recurring and recurring/metastatic ACC. MiR-139-5p, a miRNA embedded within the second intron of phospho-diesterase 2A (PDE2A) gene, is located in the 11q13 region, which is amplified in several types of human cancer including ACC (Dohna et al. 2000). Our miRNA target prediction analyses revealed that miR-139-5p could potentially target NDRG4 (Wingless-type MMTV integration site family member 4) in ACC. Very interestingly, NDRG4 has been reported as a candidate tumor suppressor gene and potential biomarker for colorectal cancer (Melotte et al. 2009) while a more recent study demonstrated that miR-139-5p was upregulated in aggressive colorectal tumors (Chang et al. 2011). MiR-376a belongs to the large 14q32 miRNA cluster, which lies within a parentally imprinted chromosomal area. MiR-376a was found upregulated in lung cancer (Liu et al. 2010). Moreover, overexpression of miR-376a conferred a tumorigenic phenotype in vitro (Teferedegne et al. 2010). A putative target of miR-376a in ACC is ELOVL7, a novel lipogenic gene encoding a long-chain fatty acid elongase, which has been shown to be overexpressed in prostate cancer cells and to be involved in prostate cancer growth (Tamura et al. 2009).

To the best of our knowledge, the levels of circulating miRNAs in ACA or ACC patients have not been reported yet. In this context, data normalization was a major challenge because there are no verified housekeeping genes existing in serum that can be used for normalization. Among the normalization strategies that have been proposed for circulating miRNA quantification, we chose the spiked-in RNA methodology using Cel-miR-39 that allows not only to monitor the efficiency of RNA extraction but also to serve as a normalization control (Mitchell et al. 2008). Furthermore, our intra- and interassay variations for the quantification of serum miRNAs were comparable to those reported by others (<10%) (McDonald et al. 2011). MiR-195 and miR-335 were dramatically decreased in the serum of ACC patients compared with ACA patients, as observed in tumor tissues. Both miRNAs displayed high diagnostic accuracy, thus demonstrating that even a single circulating miRNA is able to distinguish patients with adenomas from patients with carcinomas. Decreased circulating levels of miR-195 have been shown to differentiate hepatocellular carcinoma from chronic liver diseases patients (Qu et al. 2011) whereas miR-335 serum levels were significantly decreased in highly malignant breast tumor patients compared with controls subjects (Wang et al. 2010). Strikingly, circulating levels of miR-139-5p and miR-376a in naACC and aACC patients were not correlated with their tumor tissue

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expression in these two groups, suggesting that the predictive role of serum miRNAs may be independent of tissue specimens. Similar observations were made in lung cancer where the overexpression of let-7 miRNA family was shown to be associated with clinical outcome in tissue samples only and was not detected in the sera of the patients (Hu *et al.* 2010). We hypothesize that the release of miRNAs from ACC cells in the bloodstream might be highly selective. In particular, miR-376a could be retained within tumor cells as demonstrated for other miRNAs in breast cancer cells (Pigati *et al.* 2010). Alternatively, circulating miRNAs may not be always directly associated with the changes occurring in tumor tissues but may also reflect indirect effects (Reid *et al.* 2011). Most importantly, serum miR-483-5p was below the detection limit in naACC patients but detectable in aACC patients. As we observed a positive correlation between ACC tumor size and miR-483-5p circulating levels, we speculate that tumor burden could promote miR-483-5p release into the circulation. The mechanisms underlying miRNA release by ACC cells deserve further investigations.

Soon *et al.* (2009b) reported that miR-195 and miR-483-5p in ACC tumor tissues were associated with survival whereas Ozata *et al.* (2011) did not find a significant association between these two miRNAs and patient survival in their cohort. Our study revealed that high circulating levels of miR-483-5p and low circulating levels of miR-195 are associated with worse adrenocortical cancer prognosis. Both miRNAs seem to have a prognostic ability to identify the subpopulation of carcinoma that is at risk of postsurgical recurrence. In addition, we found that serum miR-195 markedly increased while serum miR-483-5p significantly decreased after surgical removal of primary ACC, suggesting dynamic changes of serum miRNAs in response to therapy; nevertheless, the limitation of these results is the small number of patients examined. Studies with larger cohort of patients are needed to determine whether miR-195 and miR-483-5p could be used for monitoring ACC recurrence.

In conclusion, our study demonstrates that tissue and serum miRNAs are potent biomarkers for adrenocortical cancer aggressiveness. Our data reveal that serum miRNAs could be independent predictive biomarkers for adrenocortical cancer, compared with those derived from tumor tissues. This suggests that, although tissue-based analyses are useful for understanding adrenocortical cancer biology, there is a complementary role for studying circulating miRNAs as a source of biologically and clinically relevant information.

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