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Analysis of circulating extracellular vesicle-associated microRNAs in cortisol-producing adrenocortical tumors

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Abstract

Purpose: Circulating microRNAs (miRNA) have been described in patients with adrenocortical tumors, but the expression of miRNAs in non-functioning and cortisol-producing tumors has not been yet compared. Therefore, the objective of this study was to evaluate the expression of plasma extracellular vesicle (EV)-associated microRNAs in patients with non-functioning adrenocortical adenoma (NFA), cortisol-producing adrenocortical adenoma (CPA) and cortisol-producing adrenocortical carcinoma (CP-ACC).

Methods: Preoperative plasma EV samples of 13 NFAs, 13 CPAs and 9 CP-ACCs were subjected to extracellular vesicle isolation. miRNAs were investigated by targeted quantitative real-time PCR normalized to cel-miR-39 as reference. Five miRNAs have been selected for this analysis based on previous studies including hsa-miR-22-3p, hsa-miR-27a-3p, hsa-miR-210-3p, hsa-miR-320b and hsa-miR-375.

Results: We have observed significant overrepresentation of 3 miRNAs in both CPA and CP-ACC relative to NFA: hsa-miR-22-3p ($p<0.01$ and $p<0.0001$, respectively), hsa-miR-27a-3p ($p<0.05$ in both comparisons) and hsa-miR-320b ($p<0.05$ and $p<0.0001$, respectively). Hsa-miR-320b has been significantly overrepresented in CP-ACC relative to CPA ($p<0.01$). Hsa-miR-210-3p turned out to be significantly overrepresented only in CP-ACC compared to NFA ($p<0.05$). Significant correlation was revealed between circulating miRNA concentrations and urinary free cortisol values for hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b ($p<0.0001$ for all) and cortisol after low dose dexamethasone test for hsa-miR-22-3p and hsa-miR-320b ($p<0.05$). Hsa-miR-27a-3p has been significantly stimulated by low dose dexamethasone test ($p<0.05$).

Conclusions: EV-associated miRNAs are differentially expressed in different non-functioning and cortisol-producing adrenocortical tumors.

Keywords: adrenocortical, adenoma, carcinoma, extracellular vesicle, microRNA, cortisol

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Introduction

Adrenocortical tumors are frequent, and their prevalence increases with age reaching around 10 % in the elderly in radiological series with peaks in the 5.-7. decades [1]. The vast majority of these tumors are represented by benign adrenocortical adenomas (ACA). The most common ACAs are clinically silent non-functioning adenomas (NFAs) [2]. In contrast, cortisol-producing adenomas (CPAs) result in serious morbidity and mortality associated with Cushing's syndrome [3][4]. Adrenocortical carcinoma (ACCs) is a rare (incidence 0.5-2/million/year), but aggressive tumor with poor prognosis in advanced stages [5]. In more than 80% of ACCs autonomous hormone production (mainly cortisol) is observed [6].

MicroRNAs (miRNAs) are small, non-protein coding RNA molecules that are 16-24 nucleotides long in their single-stranded mature form. More than 50 % of protein-coding human genes are predicted to be modulated by miRNAs [7]. MiRNAs, as epigenetic regulators are involved mainly in the post-transcriptional regulation of gene expression [7].

MiRNAs are found not only in tissues, but also in body fluids [8]. MiRNAs in the circulation are in part packed in actively secreted extracellular vesicles (EV) (exosomes, microvesicles, apoptotic bodies) that render them very stable [9]. Several studies (including ours) demonstrated significantly differential expression of miRNAs in benign and malignant adrenocortical tumors, both in tissue samples and blood [3][10][11][12][13]. Blood-borne (plasma or serum) circulating miRNAs could be minimally invasive biomarkers of adrenocortical malignancy as a form of liquid biopsy [10][13][14]. We have found higher diagnostic sensitivity and specificity of miRNAs isolated from EVs [14], than miRNAs isolated from whole plasma [13].

The expression of tissue miRNAs is different between hormonally active and inactive adrenocortical tumors [15], but the expression of circulating miRNAs in non-functioning and

cortisol-producing tumors has not been studied, yet. On the other hand, the expression of miRNAs appears to be modulated by hormones. In our previous study, adrenocorticotropin and dexamethasone were found to affect the expression of circulating hsa-miR-27a-3p [16]. The objective of this study was to evaluate the expression of EV-associated miRNAs in patients suffering from NFA, CPA and cortisol-producing ACCs (CP-ACC). Since urinary steroid metabolomics studies show that the vast majority of ACC, even the clinically hormonally inactive, secrete steroid hormones or their precursors [17][18], we have studied only ACC with manifest Cushing's syndrome.

Materials and methods

Patients' samples

Altogether 35 preoperative plasma samples were collected from patients with NFA (n=13), CPA (n=13) and CP-ACC (n=9). The hormonal profile was examined in all cases, and the diagnosis of hypercortisolism was based on current guidelines [4]. In the CPA and CP-ACC groups, we have included only patients having overt Cushing's syndrome. The diagnosis of ACA or ACC was determined by histological investigation in operated cases, whereas the diagnosis of non-operated ACAs was based on imaging and follow-up by abdominal computed tomography. The ENSAT (European Network for the Study of Adrenal Tumors) criteria system was used to determine the tumor stage in ACC [19]. The clinical characteristics of the three cohorts are summarized in **Table 1**. No chemo- or radiotherapy was applied in ACC-patients prior to blood collection.

We have also reanalyzed 7 plasma sample pairs from our previous study in individuals with normally functioning hypothalamo-pituitary-adrenal axis before and after low dose (1 mg) overnight dexamethasone test (LDDT) [16].

The Ethical Committee of the Hungarian Health Council endorsed these studies. Informed consent was acquired from every patient involved.

Extracellular vesicle and RNA isolation

EDTA-anticoagulated blood was collected from patients and processed for plasma isolation instantly after blood taking. Plasma was gained by centrifuging whole blood at 1000*x*g for 10 minutes at 4 ^{o}C. All extracted plasma samples were stored at -80 ^{o}C until further application. EVs were isolated from 200 µl plasma with Total Exosome Isolation (from plasma) Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Total RNA was immediately extracted from EVs using Total Exosome RNA and Protein Isolation Kit (Thermo Fisher Scientific). 5 µl of 5 nM Syn-cel-mir-39 miScript miRNA Mimic (Qiagen GmbH) was added before the addition of Acid-Phenol: Chloroform. The final total RNA elute was 50 µl and was stored at -70 ^{o}C until further use.

Analysis of miRNA expression by quantitative RT-PCR

The analysis of miRNA expression of EVs was performed using real-time qPCR. Five miRNAs (hsa-miR-22-3p (000398), hsa-miR-27a -3p (000408), hsa-miR-210-3p (000512), hsa-miR-320b (002844) and hsa-miR-375 (000564)) were studied. For data normalization, cel-miR-39 (000200) was used as reference.

Total RNA (5 µl) was reverse transcribed using specific TaqMan microRNA Assays (PN 442795, Thermo Fisher Scientific) and the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) on Proflex Base PCR System (Thermo Fisher Scientific). RT-

qPCR was performed by TaqMan Fast Universal PCR Master Mix (2x) (PN: 4352042, Thermo Fisher Scientific) on QuantStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific) according to the manufacturer's protocol (PN 4364031E, Thermo Fisher Scientific) with minor modifications (the maximum allowed CT value was set to 40). Samples were run in triplicate. Negative control reactions did not include cDNA templates

Statistical analysis

Statistical analysis of data was performed by GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA, USA). For the identification of differentially expressed miRNAs between the three groups, one-way ANOVA followed by Tukey's post Hoc test or Kruskal-Wallis test followed by Dunn test were used based on the results of Shapiro-Wilk normality test.

For data analysis, we used the -dCT (CT) method (-dCT values= - [-CT of target miRNA– -CT of internal control miRNA]) using GraphPad Prism 7.02. Correlation of basal morning cortisol levels, 24 h urinary free cortisol (UFC) and cortisol after LDDT(C-LDDT) and miRNA -dCT values were performed by Spearman correlation assay. Receiver operating characteristics (ROC) analysis was performed to test the diagnostic applicability of hsa-miR-320b between CPA and CP-ACC. For the evaluation of miRNA expression in response to LDDT, unpaired t-test was performed after Shapiro-Wilk normality test.

Results

EV-associated miRNA expression analysis of different adrenocortical tumor subtypes by RT-qPCR

35 preoperative plasma samples were evaluated by RT-qPCR analysis in order to identify differences in circulating EV-associated miRNA expression of different subtypes of adrenocortical tumors. RT-qPCR appears to be the most appropriate method to quantify the expression of extracellular miRNAs [20]. Based on previous studies, we chose to include altogether 5 human miRNAs [15][16]. The widely used synthetic spike-in control RNA cel-miR-39 was applied as reference [21].

We have observed significant underrepresentation of hsa-miR-22-3p in NFA compared with CPA (fold change (FC) 3.21) ($p<0.01$), and NFA relative to CP-ACC (FC= 7.34) ($p<0.0001$). We could not detect significant differences in its expression between CPA compared to ACC (**Figure 1a)**. Hsa-miR-27a-3p was underrepresented in NFA relative to both CPA (FC=3.55) and CP-ACC (FC=4.59) ($p<0.05$) (**Figure1b)**. We have found significantly higher expression of hsa-miR-320b in CP-ACC (FC=10.88) and CPA (FC=2.57) relative to NFA ($p<0.0001$, and $p<0.05$, respectively). Moreover, the expression of hsa-miR-320b was significantly overrepresented in CP-ACC versus CPA ($p<0.01$) (**Figure 1c)**. ROC analysis revealed an AUC of 0.8632 (sensitivity: 88.89 %, specificity: 76.92 %) for the differentiation of CP-ACC and CPA by hsa-miR-320b. Hsa-miR-210-3p was significantly overrepresented only in the CP-ACC (FC=3.1) vs NFA comparison (**Figure 1d**). Hsa-miR-375 showed no difference among different tumor types (**Supplementary Figure 1.**). Raw RT-qPCR data are presented in **Supplementary Table 1**. There were no differences in miRNA expression between males and females of the CP-ACC group.

Regarding the correlation of cortisol parameters to circulating miRNA levels (-dCT), we have observed no correlation with basal cortisol levels, but UFC levels were significantly correlated with circulating hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b ($p<0.0001$ for all) (**Figures 1e-g)**. C-LDDT concentrations were significantly associated with hsa-miR-22-3p ($p=0.0216$) and hsa-miR-320b ($p=0.0237$) **(Supplementary Table 2.**).

Hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b have been reanalyzed in sample pairs of our previous study on miRNA levels before and after LDDT. EV-associated hsa-miR-27a-3p has been significantly stimulated by 1 mg dexamethasone, whereas a tendency not reaching significance could be observed for hsa-miR-320b and hsa-miR-22-3p **(Figure 1h)**.

Discussion

Here, we report the overrepresentation of three circulating, EV-associated miRNAs in cortisol-producing adrenocortical tumors relative to non-functioning adenomas. The five miRNAs tested were selected based on three previous studies. Expression of hsa-miR-27a-3p was found to be stimulated by LDDT in our previous study [16]. We have chosen hsa-miR-22-3p and hsa-miR-320b from the study of Velazquez-Fernandez et al. on the expression of tissue miRNAs in ACA: hsa-miR-22-3p was the most overexpressed miRNA in CPA relative to normal tissue, whereas hsa-miR-320b was among the most overexpressed miRNAs in CPA vs. NFA [15]. Hsa-miR-210-3p and hsa-miR-375 were included as these were significantly differentially expressed in CPA and ACC in our previous study on tissue miRNAs [11]. We have found only one miRNA (hsa-miR-320b) to be significantly differentially expressed between CPA and CP-ACC, while the expression of the other three miRNAs was similar in benign and malignant cortisol-producing tumors. Hsa-miR-210-3p was only significantly overrepresented in CP-ACC relative to NFA, but not in CPA, thus it cannot be regarded as a

miRNA associated to cortisol-producing tumors, rather a marker of malignancy. In fact, hsa-miR-210 is the major hypoxamiR that has been shown to be overexpressed in several malignant tumors, including ACC [11][22][23].

Based on our previous studies on the diagnostic applicability of circulating miRNAs for adrenocortical malignancy isolated from whole plasma or from EVs, we have concluded that EV-derived miRNAs could be more sensitive and specific than miRNAs isolated from the whole plasma [13][14]. We have used the same protocol as in our latest study, where the kit used for exosome isolation has been verified as reliable by a parallel ultracentrifugation-based protocol including analysis of EV size distribution, and flow cytometry analysis of membrane markers [14], thus the miRNAs tested can be regarded as EV-associated.

As there is no generally accepted reference gene for raw data normalization in extracellular miRNAs studies [21], we have used the widely used synthetic spike-in control RNA cel-miR-39 as reference gene for data normalization as in our previous study [14].

No difference of miRNA expression between males and females of CP-ACC group was noted, and there are no data that the EV miRNA content in tumors would be affected by sex, thus the relative underrepresentation of males in NFA and CPA cohorts relative to CP-ACC is biologically probably irrelevant.

The cellular origin of miRNAs overrepresented in cortisol-producing adrenocortical tumor patients is unknown. Their relative abundance in hypercortisolemic patients might raise the possibility that oversecreted glucocorticoids might play a role in their overrepresentation. Our observation that the expression of three of four miRNAs has been the same between cortisol producing adrenocortical adenomas and carcinomas, might also argue for the relevance of hormonal action on the observed miRNA expression differences instead of expression changes related to tumor malignancy. In our previous study describing the

hormonal modulation of hsa-miR-27a-3p, we localized a glucocorticoid response element (GRE) within the promoter region of hsa-miR-27a-3p by *in silico* prediction [16]. Now, we could identify a potential GRE within the promoter of hsa-miR-22-3p and hsa-miR-320b, but not in hsa-miR-210-3p (TFBIND software, http://tfbind.hgc.jp). Thus, it can be assumed that hypercortisolism might be implicated in the overrepresentation of these miRNAs. This hypothesis appears to be supported by our observation that LDDT significantly stimulates EV-associated hsa-miR-27a-3p. Moreover, significant correlation with UFC was found for hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b, and with C-LDDT for hsa-miR-22-3p and hsa-miR-320b that also support the link between these miRNAs and cortisol.

Hsa-miR-27a-3p appears to be involved in several physiological and pathological conditions such as: angiogenesis, obesity, immune functioning, atherosclerosis, insulin resistance and type 2 diabetes mellitus (DM) [24]. Hsa-miR-22-3p impaired gluconeogenesis and enhanced hepatic glucose production [25]. Hsa-miR-320b was also implicated in the pathogenesis of type 2 DM [26]. It might even be possible that these miRNAs might have some role in the insulin resistance and diabetes of patients suffering from Cushing's syndrome. All overrepresented miRNAs have been associated to different tumors [27][28][29], but except for hsa-miR-210-3p, there are no data yet on their altered expression in adrenocortical malignancy [11][22].

In conclusion, our results show that EV-associated miRNAs are differentially expressed in non-functioning benign and cortisol-producing adrenocortical tumors. We hypothesize that hypercortisolism might be implicated in the overrepresentation of three of these miRNAs.

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Figure legends

Figure 1

Results of RT-qPCR analysis of (a) hsa-miR-22-3p; (b) hsa-miR-27a-3p; (c) hsa-miR-320b; normalized to the spike-in control cel-miR-39, mean±SD, *p <0.05 **p < 0.01, ***p< 0.001, ****p< 0.0001; n= 13 NFA, n= 13 CPA, n=9 CP-ACC, (One-way ANOVA followed by Tukey's post Hoc test.)

(d) Results of RT-qPCR analysis of hsa-miR-210-3p normalized to cel-miR-39, mean±SD *p <0.05, n= 12 NFA, n= 13 CPA, n=9 CP-ACC, (Kruskal-Wallis test followed by Dunn's multiple comparisons test)

Correlation between urinary free cortisol (nmol/24h) and expression of miRNAs (-dCT) for (e) hsa-miR-22-3p, (f) hsa-miR-27a-3p and (g) hsa-miR-320b, p<0.0001 n=22, Spearman rank correlation was used after Shapiro-Wilk normality test.

(h) Expression change (-dCT) of hsa-miR-22-3p, hsa-miR-27a-3p and hsa-miR-320b before and after low dose (1mg) dexamethasone suppression test in individuals having no abnormality of the hypothalamo-pituitary-adrenal axis normalized to cel-miR-39 mean±SD, *p <0.05, n=7, unpaired t-test was performed following the Shapiro-Wilk normality test. White boxes represent values before dexamethasone, grey boxes after dexamethasone administration.

Table 1

Characteristics of patients

Sample number	Tumor type	Sex	Age at blood taking	Hormonal activity	Ki-67 (%)	Weiss score	ENSAT tumor stage	Basal morning cortisol (nmol/l)	Cortisol after LDDT (C-LDDT, nmol/l)	Urinary free cortisol (UFC) (nmol/24h)
1	NFA	F	56	Non-functioning				440.6	n.d.	52.3
2	NFA	F	58	Non-functioning				494.14	n.d.	212.4
3	NFA	F	66	Non-functioning				361.98	42.21	16.8
4	NFA	F	81	Non-functioning				579.39	n.d.	n.d.
5	NFA	M	54	Non-functioning				256.6	19	n.d.
6	NFA	F	73	Non-functioning				381	n.d.	n.d.
7	NFA	F	52	Non-functioning				400	n.d.	97
8	NFA	F	63	Non-functioning				217	n.d.	n.d.
9	NFA	F	38	Non-functioning				163.82	41	413.85
10	NFA	F	60	Non-functioning				358.67	39	206.925

11	NFA	M	50	Non-functioning				609	38	n.d.
12	NFA	F	35	Non-functioning				n.d.	n.d.	n.d.
13	NFA	F	65	Non-functioning				n.d.	n.d.	188
14	CPA	F	37	Cortisol				200.9	316.45	1387
15	CPA	F	61	Cortisol				474.8	252.17	75.3
16	CPA	F	50	Cortisol				623.3	673.19	1022
17	CPA	M	62	Cortisol				676	n.d.	974
18	CPA	F	59	Cortisol				579.39	524	386.26
19	CPA	F	62	Cortisol				689.75	690	7007.86
20	CPA	F	51	Cortisol				275.9	n.d.	1682.99
21	CPA	F	55	Cortisol				n.d.	n.d.	1347
22	CPA	F	24	Cortisol				1114	510	220
23	CPA	F	43	Cortisol				420	n.d.	n.d.
24	CPA	F	47	Cortisol				n.d.	361.4	589
25	CPA	F	27	Cortisol				954	n.d.	n.d.

26	CPA	F	69	Cortisol				n.d.	103.6	n.d.
27	CP-ACC	F	67	Cortisol	n.d.	n.d.	4	1771.3	1561.5	3235.1
28	CP-ACC	F	36	Cortisol	n.d.	n.d.	4	3233.51	n.d.	15612
29	CP-ACC	F	51	Cortisol	25	9	3	742	141	n.d.
30	CP-ACC	M	62	Cortisol	30	7	2	693	796	n.d.
31	CP-ACC	M	48	Cortisol	15	8	3	728	n.d.	n.d.
32	CP-ACC	F	38	Cortisol	70	8	2	411	n.d.	n.d.
33	CP-ACC	M	58	Cortisol	30	n.d.	2	672.6	n.d.	2277.6
34	CP-ACC	F	64	Cortisol	30	5	4	1491	n.d.	10743
35	CP-ACC	M	68	Cortisol	n.d.	n.d.	1	364.5	394.81	8795

n.d.: no data, F: female, M: male, C-LDDT: cortisol after low dose dexamethasone test, UFC: 24 h urinary free cortisol

Supplementary Tables

Supplementary Table 1.

Raw data of the RT-qPCR analyses.

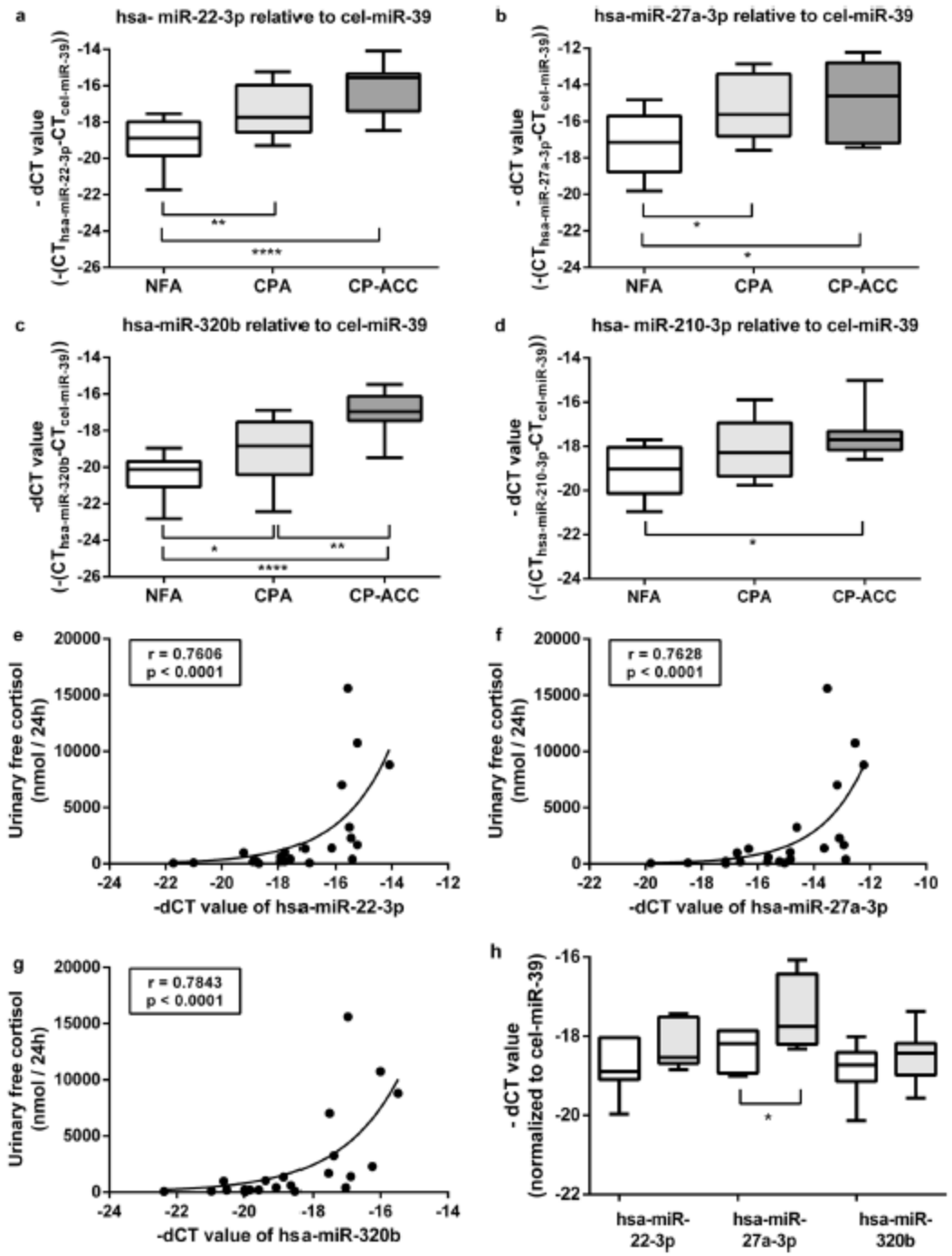
Supplementary Table 2

Spearman correlation analysis of cortisol parameters (basal morning cortisol, 24 h urinary free cortisol and cortisol after low-dose dexamethasone test) with miRNA –dCT values. Fold change and –dCT values for RT-qPCR analyses.

Supplementary Figure 1.

Results of RT-qPCR analysis for hsa-miR-375

Fig. 1.


a
hsa- miR-22-3p relative to cel-miR-39
- dCT value
(-(CThsa-miR-22-3p-CTcel-miR-39))
NFA
CPA
CP-ACC
b
hsa-miR-27a-3p relative to cel-miR-39
c
hsa-miR-320b relative to cel-miR-39
d
hsa- miR-210-3p relative to cel-miR-39
e
r = 0.7606
p < 0.0001
Urinary free cortisol (nmol / 24h)
-dCT value of hsa-miR-22-3p
f
r = 0.7628
p < 0.0001
-dCT value of hsa-miR-27a-3p
g
r = 0.7843
p < 0.0001
-dCT value of hsa-miR-320b
h
- dCT value (normalized to cel-miR-39)
hsa-miR-22-3p
hsa-miR-27a-3p
hsa-miR-320b