MicroRNA expression patterns associated with hyperfunctioning and non-hyperfunctioning phenotypes in adrenocortical adenomas

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Abstract

Background: The adrenocortical adenoma (ACA) entity includes aldosterone-producing adenoma (APA), cortisol-producing adenoma (CPA), and non-hyperfunctioning adenoma (NHFA) phenotypes. While gene mutations and mRNA expression profiles have been partly characterized, less is known about the alterations involving microRNA (miRNA) expression.

Aim: To characterize miRNA expression profile in relation to the subtypes of ACAs.

Subjects and methods: miRNA expression profiles were determined in 26 ACAs (nine APAs, ten CPAs, and seven NHFAs) and four adrenal references using microarray-based screening. Significance analysis of microarrays (SAM) was carried out to identify differentially expressed miRNAs between ACA and adrenal cortices or between tumor subtypes. Selected differentially expressed miRNAs were validated in an extended series of 43 ACAs and ten adrenal references by quantitative RT-PCR.

Results: An hierarchical clustering revealed separate clusters for APAs and CPAs, while the NHFAs were found spread out within the APA/CPA clusters. When NHFA was excluded, the clustering analysis showed a better separation between APA and CPA. SAM analysis identified 40 over-expressed and three under-expressed miRNAs in the adenomas as compared with adrenal references. Fourteen miRNAs were common among the three ACA subtypes. Furthermore, we found specific miRNAs associated with different tumor phenotypes.

Conclusion: The results suggest that miRNA expression profiles can distinguish different subtypes of ACA, which may contribute to a deeper understanding of ACA development and potential therapeutics.

Introduction

The adrenal cortex is an organ with endocrine secretion that produces three different classes of steroid hormones: mineralocorticoids (i.e. aldosterone), glucocorticoids (i.e. cortisol), and sex steroids (androgens) (¹, ²). Adrenocortical tumors may secrete any type or a combination of these steroids. Frequently, the clinical picture is associated to the secondary effects of the cortical hormones, such as Cushing’s or Conn’s syndromes. The tumors are classified as adrenocortical adenoma (ACA) or adrenocortical carcinoma (ACC). The most common functioning ACA is the aldosterone-producing adenoma (APA) or the cortisol-producing adenoma (CPA) (³). However, often ACAs are not hyperfunctioning, or at least without any obvious hormonal excess that could be clinically detected. Adrenocortical tumors could be detected incidentally when the patient is investigated for a sign or symptom not associated with the adrenal, so-called incidentaloma. When an incidentaloma is
detected, the patient should undergo screening for adrenal hormone overproduction (4). A tumor without hormonal overproduction, which is small (≤4 cm) and without any radiological sign of malignancy, is usually left untreated. Several consensus guidelines have tried to assist in this decision algorithm (5). A tumor that has reached a certain critical size (>4–6 cm) is usually surgically removed due to its potentially higher risk for being or becoming malignant. However, if this tumor displays benign features and does not have any detectable hormonal overproduction, it is usually categorized as a non-hyperfunctioning adenoma (NHFA).

ACAs are characterized by few numerical genetic alterations. Activating somatic mutations of the KCN5 gene encoding a potassium channel are found in a subset of APA (6). Recently, other mutations have been reported in ATP1A1 and ATP2B3 that lead to APA and secondary hypertension. These somatic mutations are present in 5.2 and 1.6%, respectively, in sporadic APA.

A large part of the transcriptome consists of transcribed non-coding RNA, including microRNA (miRNA). These are small single-stranded RNA molecules, which are ~20–25 nucleotides long in their mature form (7). In the last decade, it has been demonstrated that miRNAs play an important role in the regulation of gene expression (7), acting both on the transcriptional and translational level. It has been documented that these molecules are related to different functions such as tissue development, cell differentiation, apoptosis, invasiveness, chemosensitivity, and particularly tumorigenesis. Several global miRNA expression studies using microarray platforms have demonstrated that subsets of miRNAs are differentially expressed between ACA and ACC. However, miRNA profiles among ACA subtypes remain unclear.

Our aim in this study is to determine whether miRNA profiles are associated with functional phenotypes of ACAs. For this purpose, miRNA expression was determined in a panel of ACAs and adrenal references and evaluated in relation to the phenotype.

**Subjects and methods**

**Clinical subjects**

Our cohort was composed of 16 APAs, 13 CPAs, and 14 NHFAs (Supplementary Table 1, see section on supplementary data given at the end of this article). Ten normal non-tumorous adrenal cortical tissues collected from patients operated by nephrectomy were used as reference (8). All samples had been collected and snap-frozen in liquid nitrogen, and stored at −80 °C in the Endocrine Biobank of the Karolinska University Hospital until its definitive use. The Local Ethical Committee for human research approved the collection and use of tissue samples, and informed consents were obtained from all patients. Histopathological diagnosis was established following the WHO classification (9). The clinical classification of aldosteronoma (APA), CPA, or NHFA was based on established clinical and biochemical signs (10). A representative section from each specimen was histopathologically re-evaluated for the verification of high content of tumor cells or non-cancer cells. Clinical data were collected from medical records.

**RNA extraction and purification**

Total RNA was isolated using mirVana miRNA isolation kit (Applied Biosystems/Ambion), following the procedures recommended by the manufacturer. The isolated RNA was quantified by NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

**Microarray-based miRNA profiling and analyses**

miRNA profiling was carried out on 26 ACAs (nine APAs, ten CPAs, and seven NHFAs) and four adrenal references using Agilent’s human miRNA microarray system (Agilent Technologies, Santa Clara, CA, USA), which has been described in Ozata et al. (8). This platform includes probes for 903 human miRNAs based on the miRBase release, version 14. In brief, ~120 ng of total RNA were labeled with Cyanine 3, hybridized onto arrays for 18–20 h at 55 °C, and scanned using microarray Scanner G565BA (Agilent Technologies). Spot images were processed by Feature Extraction Software v10.7.3.1 (Agilent Technologies). We used Cluster 3.0 Software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) for data normalization and median centering.Normalized miRNAs with >50% missing values were excluded for hierarchical clustering and significance analysis of microarrays (SAM in Multi Experiment Viewer (MeV) 4.5.1) analyses. Unsupervised hierarchical clustering using Spearman’s rank order correlation and complete linkage were carried out in Cluster 3.0, and the results were visualized by Java TreeView v1.1.6r2. SAM analysis was applied to determine the most significant differentially expressed miRNAs between the two groups. False discovery rates (FDRs) were estimated with 1000 permutations or the maximum number permissible, with a
median FDR threshold of \( \leq 0.05 \) or 5\%. The microarray data are available at NCBI Gene Expression Omnibus (GEO) with the accession number GSE22816.

Quantitative RT-PCR

All 43 ACAs and ten adrenal references were included in the quantitative RT-PCR (qRT-PCR) analyses. Expression of 14 selected mature miRNAs, i.e. MIR-21 (ID 000397), MIR-186 (ID 002105), MIR-497 (ID 001043), MIR-210 (ID 000512), MIR-10b (ID 002218), MIR-320b (ID 002844), MIR-320c (ID 241053_mat), MIR-30e (ID 000422), MIR LET-7f (ID 000382), MIR-139-5p (ID 002289), MIR-195 (ID 000494), MIR-1274b (ID 002884), MIR-34a (ID 000426), and MIR-520d-3p (ID 002743) was measured by TaqMan qRT-PCR method (Applied Biosystems). Four of these miRNAs, i.e. MIR-21, MIR-210, MIR-195, and MIR-497, were quantified in the same samples in our previous study (8), for comparison with carcinomas. RNU6B (ID 001093) was used as an endogenous control for normalization of miRNA expression level. Relative expression quantification was carried out by the \( \Delta \Delta C_T \) method and calculated as \( 2^{-\Delta C_T} \). All reactions were run in triplicates.

Statistical analysis

All analyses were evaluated using the IBM SPSS Statistics Software, version 20.0 (IBM, Inc., Chicago, IL, USA) and MS Excel 2011 for Mac, version 12.2.6. Unpaired Student’s t-test for independent samples and one-way ANOVA were carried out to compare differences in miRNA expression levels between studied groups. Other variables were analyzed according to their scaling, using \( \chi^2 \) test for categorical variables and t-test for dimensional variables. All hypotheses were two-sided tests, considering any \( P \) value \( \leq 0.05 \) or 5\% as statistically significant.

### Results

We included 43 adenomas (16 APA, 13 CPA, and 14 NHFA) and ten normal adrenal cortices in this study. The clinical data of the adenoma cases are detailed in Supplementary Table 1 and summarized in Table 1. Two patients (4.6\%) died during follow-up for reasons not related to their adrenal disease. None of the patients was diagnosed with metastasis or tumor recurrence during follow-up. Following adrenalectomy, one patient persisted with arterial hypertension, while the remaining APA cases were free from biochemical and clinical signs of the disease postoperatively. CPA patients were postoperatively all free from typical symptoms related to Cushing’s syndrome. miRNA profiling was previously carried out in our laboratory to compare expression levels between adrenocortical carcinomas and adenomas or adrenal cortices (8). In this study, we further investigated whether global miRNA expression profiles could distinguish between different subtypes of ACAs.

**miRNA profiles of hyperfunctioning and non-hyperfunctioning ACAs**

Using the 211 miRNAs that passed the filtering criteria, we carried out hierarchical clustering analysis for 26 ACA representing three different tumor subtypes (nine APA, ten CPA, and seven NHFA), in an unsupervised manner. As shown in Fig. 1A, the clustering algorithm revealed four major clusters: clusters 1 and 2 composed of all APA tumors, and the majority of CPAs were found in clusters 3 and 4, with the NHFA tumors being distributed across all clusters. By excluding the NHFA tumors from the unsupervised clustering, we found a better separation between the APAs and CPAs (Fig. 1B). Interestingly, we noted two separate clusters of APA by unsupervised clustering analysis (Fig. 1A and B), suggesting two subgroups of APA tumors. However, these clusters were not associated with the KCNJ5 mutation status.

### Table 1

Summary of the clinical features for the 43 adrenocortical adenomas included in this study.

<table>
<thead>
<tr>
<th>Tumor phenotype</th>
<th>n</th>
<th>Gender (female/male)</th>
<th>Age at diagnosis (years)*</th>
<th>Tumor size (cm)*</th>
<th>Follow-up time (months)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-hyperfunctioning adenoma (NHFA)</td>
<td>14</td>
<td>8/6</td>
<td>63 (42–66)</td>
<td>4 (2.5–5.3)</td>
<td>96.5 (12–216)</td>
</tr>
<tr>
<td>Aldosterone-producing adenoma (APA)</td>
<td>16</td>
<td>11/5</td>
<td>48 (16–79)</td>
<td>1.8 (0.9–4.7)</td>
<td>30 (15–258)</td>
</tr>
<tr>
<td>Cortisol-producing adenoma (CPA)</td>
<td>13</td>
<td>12/1</td>
<td>60 (27–81)</td>
<td>4 (1.5–6.5)</td>
<td>32 (12–231)</td>
</tr>
</tbody>
</table>

n, number.

*Values are expressed in medians and range.
Differential miRNA expression patterns between ACAs and adrenal references

In order to identify the most significant differentially expressed miRNAs between the ACAs and adrenal references, we applied SAM analysis that identified a total of 43 differentially expressed miRNAs in ACAs with a FDR of 0. Forty of the selected miRNAs were over-expressed and three miRNAs were under-expressed in ACAs (Supplementary Table 2, see section on supplementary data given at the end of this article).

To further investigate the miRNA expression differences between tumor subtypes of ACAs and adrenal references, we compared the expression profiles of individual tumor subtype with its normal counterpart through SAM analysis. The results showed that there were more over-expressed miRNAs than under-expressed miRNAs in all tumor subtypes (Fig. 2 and Supplementary Table 2). Among the differentially expressed miRNAs, 14 miRNAs were common for the three adenomatous subtypes: nine were highly expressed and five had lower expression in all ACA subtypes (Fig. 2). Among the hyperfunctioning ACAs, many differentially expressed miRNAs were distinctive to specific tumor subtypes and only a single miRNA (MIR-376a) was common for both CPA and APA. However, many deregulated miRNAs were common between CPA and NHFA, and only three miRNAs were unique to NHFA (Fig. 2). The results are consistent with the clustering data, where most of the NHFAs were grouped closely with the CPAs (Fig. 1A).

Figure 1
Unsupervised clustering of miRNA expression data determined by microarray for (A) all nine APAs, ten CPAs, and seven NHFAs and (B) nine APAs and ten CPAs only. Samples were clustered using the Spearman’s rank correlation and complete linkage. Red and green colors represent relatively high and low expression respectively. Gray indicates missing values.
miRNA expression signatures for specific ACT phenotypes

To further explore the miRNA expression differences among the ACA subtypes, we directly compared the miRNA expression profiles among the three tumor subtypes. Using SAM analysis, we found 50 under-expressed miRNAs in APA when compared with CPA. Twenty-three miRNAs were found under-expressed in APA when compared with NHFA. Eight miRNAs had higher expression in CPA than NHFA (Supplementary Table 2).

Validation of miRNA microarray data by qRT-PCR

In order to verify the array data, we evaluated the expression of 14 miRNAs in an extended cohort of 43 adenomas (16 APA, 13 CPA, and 14 NHFA) and ten adrenal references by qRT-PCR. The miRNAs were selected based on their high scores in the SAM analysis for different comparisons and their associations with biological processes such as steroidogenesis or carcinogenesis. Ten miRNAs (MIR-21, MIR-497, MIR-10b, MIR-30e, MIR-139-5p, MIR-210, MIR-195, MIR-520d-3p, and MIR-34a) were confirmed as statistically significant between all adenomas and the adrenal cortex references (Fig. 3).

Among the tumor subtypes, two miRNAs (MIR-21 and MIR-10b) were significantly differentially expressed between APA and NHFA or CPA. APA samples also showed a higher expression of MIR LET-7f, MIR-210, and MIR-139-5p when compared with CPA, NHFA, and adrenal cortex references respectively (Fig. 4).

Discussion

ACAs display a variable spectrum of clinical phenotypes depending on the type of hormone secreted by the tumor. Despite several studies attempting to characterize the gene expression profiles of these benign tumors (12, 13, 14, 15), the molecular basis of these tumors has not yet been fully understood. Previous miRNA-profiling studies in ACT focused mainly on differential miRNA expression associated with malignancy (8, 11, 16, 17, 18); their expression patterns in benign tumors remain unclear. Here, we characterized miRNA expression profiles of three common subtypes of ACA and determined the common and different deregulated miRNAs among the tumor subtypes.

miRNA expression patterns between ACAs and adrenal references

Using SAM analysis, we identified a set of 43 differentially expressed miRNAs among all adenomas and their normal counterparts. Among them, ten were validated in a larger cohort by qRT-PCR. In our previous study, four of these miRNAs (MIR-21, MIR-210, MIR-195, and MIR-497) were also found differentially expressed between ACCs and adrenal references (8). Both MIR-21 and MIR-210 are commonly over-expressed in many tumor types. Besides MIR-21 and MIR-210, three other validated miRNAs (MIR-195, MIR-497, and MIR-139-5p) were also found differentially expressed between ACC and ACA (8, 18, 19). Notably, MIR-195 and MIR-497 levels are equally low.
These findings suggest that MIR-139-5p may have different functions in ACA and ACC.

Although MIR-21 and MIR-210 expressions were significantly higher in both adenomas and carcinomas as compared with adrenal reference (Fig. 4), their expression levels were still significantly higher in carcinomas than in adenomas; this suggests that MIR-21 and MIR-210 may be involved in both benign and malignant processes and their excessive levels may contribute to malignant development. Similar results were reported by Tombol et al. (20), although these authors found MIR-210 to be upregulated in ACC but downregulated in CPA when compared with normal adrenals and hormonally inactive tumors. MIR-210 is also known to be regulated by hypoxia-inducible factors and plays important roles in tumor angiogenesis, stem cell function, and cell-cycle regulation, and is implicated in tumor progression (21, 22, 23).

**Figure 3**
Boxplots showing the relative expression levels of individual miRNAs in adrenal references and ACAs determined by qRT-PCR. Two-tailed unpaired t-test was used to assess the statistical significances between the two groups and P < 0.05 were considered as significant.

in both ACCs and adrenal cortices as compared with ACAs, suggesting that these two miRNAs may play important roles in the pathogenesis of ACA. In ACC, we have previously demonstrated that MIR-195 and MIR-497 have tumor suppressive functions by reducing cell growth and promoting cell apoptosis (8). Their functional roles in ACA have yet to be determined. Chabre et al. (19) reported higher expression of MIR-139-5p observed in aggressive ACCs than in non-aggressive ACCs or ACAs.

**Figure 4**
Boxplots showing individual miRNA expressions determined by qRT-PCR in adrenal cortices, and the NHFA, APA, and CPA subgroups. Two-tailed unpaired t-test was used to assess the statistical significances between the two groups and P < 0.05 were considered as significant.
miRNA expression patterns among different subtypes of ACA

In this study, we demonstrate that miRNA expression patterns are distinct between APA and CPA subtypes, based on the following findings: i) unsupervised clustering analysis revealed separate clusters between APA and CPA and ii) SAM analysis identified a large number of miRNAs unique to individual subtypes. The clustering analysis also suggests two subgroups of APA, reflecting clinical and/or genetic heterogeneity of this tumor subtype. Notably, these two APA subgroups did not significantly differ regarding gender, age, tumor size, biochemical features (such as serum potassium or hormonal levels), or KCNJ5 mutation status (data not shown).

In contrast to both types of hyperfunctioning tumors, the NHFA tumors did not have many unique miRNAs. However, they share many common deregulated miRNAs with CPA, suggesting the biological similarities between CPA and NHFA. This finding is also consistent with the clustering data, where the majority of NHFA tumors were grouped closely with the CPA tumors. In line with our findings, recently two subgroups of CPA were identified, in which one of the subgroups was closely clustered with the NHFA tumors based on gene expression profiles (24). It is also worth noting that 5–20% of NHFA patients may have mild cortisol secretion without causing typical symptoms of hypercortisolism (25), further supporting the similarities between these two tumor subtypes.

Using microarray and qRT-PCR approaches, we identified four differentially expressed miRNAs among the ACA subtypes. The expression levels of MIR-21 and MIR-10b are clearly distinct between APA and CPA or NHFA, suggesting that these two miRNAs can be useful to distinguish APA from the other two subtypes. MIR LET-7f can distinguish APA from CPA but not NHFA, while MIR-139-5p is a differential marker between APA and NHFA. MIR-21 is commonly over-expressed in a variety of tumor types (26, 27, 28, 29), and it is known to function as an oncogenic miRNA by targeting the tumor suppressors PTEN (30) and PDCD4 (31). Besides its involvement in malignancy, MIR-21 has also been shown to have a role in myocardial disease (32), immunity (33), and development (34). In ACC cells, MIR-21 has been shown to increase cell proliferation and aldosterone secretion (35). Given that MIR-21 expression levels in APA samples are similar to adrenal references and relatively lower than the other two tumor subtypes, it is plausible that MIR-21 may have other function(s) in APA cells. Similar to MIR-21, MIR-10b, MIR-139-5p, and MIR LET-7f have been reported to be involved in ACC development, progression, and aggressiveness (19, 36, 37). In our study, we found differential expression of these miRNAs compared with normal adrenal and between ACA subtypes, mainly APA. In addition, MIR-24 has been reported as a regulator of aldosterone and cortisol production by targeting the CYP11B1 and CYP11B2 genes (38). In our study, we found higher MIR-24 expression determined by microarray in CPA vs APA but no significant differences between normal adrenal and the tumor groups (Supplementary Fig. 1, see section on supplementary data given at the end of this article), which may be due to the limited number of samples studied. Four miRNAs were selected for further verification because of their associations with steroidogenesis, i.e. MIR-21 (35), MIR-210 (39), MIR-320, and MIR-520 (40). Of these, MIR-21 and MIR-210 were further verified by qRT-PCR as differentially expressed between sample subgroups. Besides this, there is limited information published about the role of miRNAs in human steroidogenesis. Further investigation is necessary to address the role of these miRNAs in aldosterone/cortisol synthesis and adrenocortical tumor development.

In summary, we demonstrate that APA, CPA, and NHFA share common and different miRNA expressions among the three ACA subtypes. We also identified specific miRNAs associated with each tumor subtype.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-13-0817.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
D Velázquez-Fernández, S Caramuta, D M Özata, C Larsson, W-O Lui, and J Zedenius conceived and designed the experiments and the main research hypothesis; D Velázquez-Fernández, S Caramuta, D M Özata, and M Lu performed the experiments; D Velázquez-Fernández, S Caramuta, D M Özata, and W-O Lui performed the statistical analysis and data representation; A Höög, M Bäckdahl, C Larsson, W-O Lui, and J Zedenius analyzed the draft manuscript and corrected the style and proper contents of this paper; A Höög evaluated and contributed to the histopathological analysis.
information of included cases; D Velázquez-Fernández, M Bäckdahl, C Larsson, W-O Lui, and J Zedenius contributed and analyzed the clinical data of included cases; M Bäckdahl reviewed the clinical file of every included patient and the clinical follow-up and final status; and C Larsson, W-O Lui, and J Zedenius supervised all the steps of this work including the experiments. All the aforementioned authors fully contributed to the reading, writing, and approval of the final version of this manuscript.

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References


