MicroRNAs Differentially Expressed in ACTH-Secreting Pituitary Tumors

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Context: MicroRNAs (miRNAs) are small noncoding RNAs, functioning as antisense regulators of gene expression by targeting mRNA and contributing to cancer development and progression. More than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites of the genome.

Objective: The aim of the study was to analyze the differential expression of let-7a, miR-15a, miR-16, miR-21, miR-141, miR-143, miR-145, and miR-150 in corticotropinomas and normal pituitary tissue and verify whether their profile of expression correlates with tumor size or remission after treatment.

Material and Methods: ACTH-secreting pituitary tumor samples were obtained during transphenoidal surgery from patients with Cushing disease and normal pituitary tissues from autopsies. The relative expression of miRNAs was measured by real-time PCR using RNU44 and RNU49 as endogenous controls. Relative quantification of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Results: We found underexpression of miR-145 (2.0-fold; $P = 0.04$), miR-21 (2.4-fold; $P = 0.004$), miR-141 (2.6-fold; $P = 0.02$), let-7a (3.3-fold; $P = 0.003$), miR-150 (3.8-fold; $P = 0.04$), miR-15a (4.5-fold; $P = 0.03$), miR-16 (5.0-fold; $P = 0.004$), and miR-143 (6.4-fold; $P = 0.004$) in ACTH-secreting pituitary tumors when compared to normal pituitary tissues. There were no differences between miRNA expression and tumor size as well as miRNA expression and ratio of remission after surgery, except in patients presenting lower miR-141 expression who showed a better chance of remission.

Conclusion: Our results support the possibility that altered miRNA expression profile might be involved in corticotrophic tumorigenesis. However, the lack of knowledge about miRNA target genes postpones full understanding of the biological functions of down-regulated or up-regulated miRNAs in corticotropinomas. **(*J Clin Endocrinol Metab* 94: 320–323, 2009)**

MicroRNAs (miRNAs) are an abundant class of endogenous small RNA molecules involved in temporal and tissue-specific eukaryotic gene regulation (1). miRNAs act as negative regulators of the protein-coding gene expression controlling multiple biological processes, including stem cell division (2), and apoptosis (3). Several human cancers are associated with altered miRNA expression (4. In addition, miRNAs regulate the expression of oncogenes and tumor suppressor genes (5), suggesting that miRNAs might play a role in tumorigenesis (6).

Cushing's disease (CD) is a disorder caused by autonomous ACTH-secreting pituitary adenoma. The pathogenesis of CD remains unknown; however, activation of cellular protooncogenes and/or loss of function of tumor suppressor genes have been implicated in the initiation of corticotroph tumors (7). Using the microarray technique, several deregulated miRNAs have

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Abbreviations: CD, Cushing's disease; Ct, cycle threshold; IHC, immunohistochemistry; miRNA, microRNA; q-PCR, real time-PCR; SAGE, serial analysis of gene expression.

been involved not only in pituitary cell proliferation and apoptosis, but also in neoplastic transformation (8). miR-15a and miR-16 were expressed at lower levels in GH- and PRL-secreting pituitary adenomas compared with normal pituitary tissue, were inversely correlated with tumor diameter, and were directly correlated with secretion of the antineoplastic cytokine p43 (9). Definitive evidence linking miRNAs with the development of pituitary cancer is still scarce. Therefore, in this study, we investigated the expression of several miRNAs, including let-7a, miR-15a, miR-16-1, miR-21, miR-141, miR-143, miR-145, and miR-150 in corticotropinomas compared with normal pituitary tissues. We also verified whether miRNA expression in ACTH-secreting pituitary tumors correlates with the tumor size or remission after transphenoidal surgery.

Patients and Methods

The study was approved by the Institutional Review Board of the University Hospital of School of Medicine of Ribeirao Preto, University of Sao Paulo, and all participants gave informed consent. Fourteen patients were diagnosed as having CD using standard tests of pituitary-adrenal function, including plasma ACTH levels, low- and high-doses of dexamethasone suppression tests, ovine CRH test, inferior petrosal sinus sampling after administration of ovine CRH, when appropriated, and image studies.

ACTH-secreting pituitary tumor samples were obtained during transphenoidal surgery, snapped-frozen and/or processed for routine histopathological examination, and stored at -70 C. For miRNA expression study, tumoral samples were microdissected by an experienced pathologist to separate any nontumoral tissue. As control, seven normal pituitaries were obtained during autopsies from subjects who had suffered accidental death and presented no previous evidence of any endocrine disease or other abnormalities. All samples were disrupted using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland), and total RNA was isolated by TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA).

We had previously performed two cDNA serial analysis of gene expression (SAGE) libraries obtained from normal pituitaries or corticotropinomas, using I-SAGE kit (Invitrogen Life Technologies, Inc.), as previously described (10). Sequences were further analyzed using SAGE2000 software. The annotation of tag sequences was based on the SAGEmap database (http://www.ncbi.nlm.nih.gov/SAGE). Statistical analysis was carried out with the H2G software (http://gdm.fmrp.usp.br) to evaluate hyper- and hypoexpressed genes. Then, let-7a, miR-15a, miR-16, miR-21, miR-141, miR-143, miR-145, and miR-150 were identified based on a list of miRNAs differentially expressed in ACTH-secreting pituitary tumor library in comparison with a normal pituitary library. Primers and probe mix of the TaqMan* MicroRNA Assay for each miRNA and endogenous controls (RNU44 and RNU49) were purchased from Applied Biosystems (Foster City, CA). Each miRNA was validated by real time-PCR (q-PCR) using a 7500 Real-Time PCR System (Applied Biosystems, Singapore). Reactions were incubated in a 96-well optical plate at 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 10 min. The cycle threshold (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The Ct data were obtained using default threshold settings.

Data were presented as $\log_{10}$ of relative quantity of target miRNA, normalized by the endogenous controls (RNU44 and RNU49) Ct-median expression, and calibrated by ΔCt-median value obtained from all normal pituitary tissue. Relative quantification of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (11). Fold change of the expression of each miRNA observed in corticotropinomas in relation to normal pituitaries was determined by the median of $2^{-\Delta\Delta Ct}$ values of corticotropinomas related to median of $2^{-\Delta\Delta Ct}$ values of normal pituitaries.

The expression of ACTH-secreting pituitary tumor samples of each miRNA is presented as mean ± SD, median, and range. Statistics were carried out using the Mann-Whitney test for continuous variables or Fisher's exact test for categorical data. To analyze the association of each miRNA expression and tumor size or remission after transphenoidal surgery, we divided tumors samples in two subgroups (<50% or ≥50%) based on the $2^{-\Delta\Delta Ct}$ values. Data were analyzed by GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA), and differences were considered significant at $P < 0.05$.

Results

Table 1 shows the clinical findings and follow-up of patients with CD. Magnetic resonance imaging showed no tumor evidence in patients 1, 4, 6, 7, and 8; macroadenoma was observed only in patient 11, whereas all other patients showed microadenoma. Postoperative undetectable cortisol serum level (<50 nmol/liter) during the first days after surgery was used as a marker to define CD remission. Based on this criterion, we observed nine patients

TABLE 1. Clinical findings and follow-up of patients with ACTH-secreting pituitary tumors

Patient no.	Age (yr)	Gender	Tumor size on MRI (cm)	Follow-up (months)	IHC	Remission
1	52	F	No tumor evidence	96	[a]	No
2	51	F	0.5	35	ACTH +	Yes
3	20	F	0.8	34	ACTH +	No
4	15	M	No tumor evidence	24	Hyperplasia, LH+ diffuse, ACTH+	Yes
5	24	F	0.6	24	ACTH +	Yes
6	59	F	No tumor evidence	24	[a]	No
7	12	M	No tumor evidence	53	ACTH +	Yes
8	39	F	No tumor evidence	77	[a]	Yes
9	33	M	0.8	57	ACTH +	No
10	27	F	0.8	27	ACTH +	Yes
11	31	F	1.5	78	ACTH +	No
12	45	F	1.0	31	ACTH +	Yes
13	25	F	1.0	28	ACTH +	Yes
14	36	F	1.0	33	ACTH +	Yes

F, Female; M, male; MRI, magnetic resonance imaging.

[a] No neoplasia in the histological sample and no inclusion on miRNA analysis.

(64.3%) with remission without recurrence in a period of follow-up of 33.5 months (24 to 96 months). Five patients (35.7%) showed no remission after transphenoidal surgery and were submitted to ketoconazole treatment and/or adrenalectomy. Positive immunohistochemistry (IHC) for ACTH in tissue samples was observed in 11 patients (78.6%), and three patients showed no neoplasia in the histological sample; therefore, to avoid any inaccurate results regarding miRNA expression, we removed these three samples from the analysis.

Table 2 shows the mean-ΔCt ± SD, the median, and the range of each miRNA obtained from corticotropinomas normalized by the ΔCt-median of normal pituitaries and also the fold-change of the expression of each miRNA observed in corticotropinomas in relation to normal pituitaries. We observed underexpression of miR-145 (2.0-fold; $P = 0.04$), miR-21 (2.4-fold; $P = 0.004$), miR-141 (2.6-fold; $P = 0.02$), let-7a (3.3-fold; $P = 0.003$), miR-150 (3.8-fold; $P = 0.04$), miR-15a (4.5-fold; $P = 0.03$), miR-16 (5.0-fold; $P = 0.004$), and miR-143 (6.4-fold; $P = 0.004$) in corticotropinomas compared with normal pituitaries. There was no difference between the expression of let-7a, miR-15a, miR-16, miR-21, miR-143, miR-145, and miR-150 and tumor size as well as in the expression of these miRNAs and the ratio of remission after transphenoidal surgery. However, patients who presented lower miR-141 expression showed a better chance of remission (odds ratio, 32; 95% confidence interval, 1.5–656.5; $P = 0.02$).

Discussion

We demonstrated underexpression of let-7a, miR-15a, miR-16, miR-21, miR-141, miR-143, miR-145, and miR-150 in corticotropinomas compared with normal pituitary tissues, which were validated by q-PCR. These findings, using SAGE analysis, are in accordance with recent studies using microarray and Northern blot, which have shown that several miRNAs are aberrantly expressed in cancer (6), including endocrine cancer (8, 9, 12).

MiR-15a and miR-16 genes are located in chromosome region 13q14. Losses in 13q were detected in secreting and nonsecreting pituitary adenomas, including corticotropinomas, indicating that a putative tumor suppressor gene(s) on 13q could play a role in the development of pituitary adenomas (13). miR-15a and miR-16 genes were underexpressed in our series of corticotropinoma. The first study describing reduction of miR-15a and miR-16 expression in samples of GH- or PRL-pituitary-secreting adenomas was performed by Bottoni *et al.* (9). Different from our finding on corticotropinomas, where no correlation was found between reduced expression of miR-15a and miR-16 and tumor size or remission, these authors demonstrated an inverse correlation of reduced expression of these miRNAs and tumor diameter. Altogether, these data suggest a role of reduced expression of miR-15a and miR-16 in the pathogenesis of pituitary tumors, but not in a pituitary-specific cell lineage.

Underexpression of let-7a, miR-143, and miR-145 had been previously described in breast cancer (14), lung cancer (15), and colorectal neoplasia (16). We observed, for the first time, an underexpression of these miRNAs in 11 samples of corticotropinomas, suggesting their involvement in carcinogenesis. It is important to point out that decreased miR-143 expression may be directly involved in carcinogenesis through activation of the MAPK cascade via ERK5 (17). Furthermore, miR-145 potential target genes encode proteins with potential oncogenic functions, such as *MYC*, *KRAS*, *FOS*, *YES*, and *FLI*, as well as cyclin D2, and MAPK transduction proteins (14).

We found reduced expression of miR-150 and miR-21 in corticotropinomas. Previous studies have demonstrated overexpression of miR-150 in hematopoietic progenitor/stem cells (18) and overexpression of miR-21 in solid tumors (4) and cell lines (19, 20). Suppression of mir-21 by antisense oligonucleotides or miR-21 knockdown was associated with increased apoptotic activity and inhibition of tumor cell growth, probably by down-regulation of the target tumor suppressor genes (20). Conversely, our data suggest that in corticotrophs miR-150 and miR-21 may act by up-regulation of the target tumor genes, acting as a tumor suppressor gene.

A recent study analyzed the entire miRNAome in 32 pituitary adenomas and in six normal pituitary samples by microarray and q-PCR, showing 30 miRNAs differentially expressed. Among these, miR-30a, -30b, -30c, and -30d were strongly hyperexpressed in four ACTH-secreting pituitary adenomas, suggesting that miRNA expression profile in this class of adenomas was clearly different from other pituitary tumor histotypes, probably due to the early determination of corticotroph lineage during pituitary cytodifferentiation (8).

Although among all miRNAs identified in our study there are several involved in cell proliferation and apoptosis, we observed no association with their expression in ACTH-secreting pituitary tumors and tumor size. On the other hand, the subset of patients

TABLE 2. miRNA expression in ACTH-secreting pituitary tumors

miRNA	Normal (mean ΔCt)	Tumor (mean ΔCt)	Tumor (mean $2^{-\Delta\Delta Ct}$ ± SD, median, range)	Tumor/normal fold
let-7a	−3.55 ± 0.62	−1.57 ± 1.26	0.35 ± 0.07, 0.29 (0.05 to 1.04)	−3.3 ($P = 0.01$)
miR-15a	2.57 ± 0.80	4.13 ± 1.66	0.54 ± 0.18, 0.20 (0.06 to 2.33)	−4.5 ($P = 0.05$)
miR-16	−3.95 ± 1.06	−1.50 ± 1.29	0.36 ± 0.09, 0.21 (0.05 to 1.30)	−5.0 ($P = 0.002$)
miR-21	−2.54 ± 0.84	−0.66 ± 2.00	0.52 ± 0.13, 0.44 (0.02 to 1.84)	−2.4 ($P = 0.008$)
miR-141	−1.61 ± 1.49	1.24 ± 3.54	0.56 ± 0.22, 0.35 (0.01 to 3.10)	−2.6 ($P = 0.02$)
miR-143	0.69 ± 1.23	3.34 ± 2.00	0.25 ± 0.06, 0.13 (0.01 to 0.76)	−6.4 ($P = 0.05$)
miR-145	0.04 ± 1.13	1.25 ± 1.43	0.54 ± 0.13, 0.56 (0.05 to 1.88)	−2.0 ($P = 0.04$)
miR-150	1.17 ± 1.04	0.01 ± 1.44	0.57 ± 0.17, 0.31 (0.05 to 1.98)	−3.8 ($P = 0.04$)

Normal, Normal pituitary tissue; tumor, ACTH-secreting pituitary tumors.

with ACTH-secreting pituitary tumors who expressed lower miR-141 had a higher chance of remission after transphenoidal surgery, suggesting a possible role of the miR-141 in the regulation of pituitary genes involved in tumor growth and tumor local invasion, which can influence the ratio of remission after surgery.

In conclusion, our results obtained from pituitary tumors of patients with CD support the possibility that altered miRNA expression profile might be involved in corticotrophic tumorigenesis. However, the lack of knowledge about miRNA target genes postpones full understanding of the biological functions of miRNAs. Therefore, further studies are needed to predict miRNA target genes for either down-regulated or up-regulated miRNAs in pituitary adenomas.

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