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Identification of Differentially Expressed MicroRNAs by Microarray: A Possible Role for MicroRNA Genes in Pituitary Adenomas

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MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression by targeting mRNA. It has been demonstrated that miRNA expression is altered in many human cancers, suggesting that they may play a role in human neoplasia. To determine whether miRNA expression is altered in pituitary adenomas, we analyzed the entire miRNAome in 32 pituitary adenomas and in 6 normal pituitary samples by microarray and by Real-Time PCR. Here, we show that 30 miRNAs are differentially expressed between normal pituitary and pituitary adenomas. Moreover, 24 miRNAs were identified as a predictive signature of pituitary adenoma and 29 miRNAs were able to predict pituitary adenoma histotype. miRNA expression could differentiate micro- from macro-adenomas and treated from non-treated patient samples. Several of the identified miRNAs are involved in cell proliferation and apoptosis, suggesting that their deregulated expression may be involved in pituitary tumorigenesis. Predictive miRNAs could be potentially useful diagnostic markers, improving the classification of pituitary adenomas. J. Cell. Physiol. 210: 370–377, 2007. © 2006 Wiley-Liss, Inc.

MicroRNAs (miRNAs) are a class of small non-coding RNAs involved in temporal and tissue-specific eukariotic gene regulation (Lagos-Quintana et al., 2002). Mature miRNAs are 19–25 nucleotides (nt) long and are generated from endogenous hairpin-shaped transcripts. Hundreds of miRNA genes have been discovered, that likely function as antisense regulators of mRNAs (Ambros, 2001). miRNAs bind to 3′-untranslated region of target mRNAs, causing block of translation or mRNA degradation (Bartel, 2004). miRNAs may be involved in several cellular processes, including differentiation, proliferation, and death (Croce and Calin, 2005).

Recently, it has been shown that several human cancers, such as breast cancer (Iorio et al., 2005), leukemia (Calin et al., 2002, 2004a,b; Cimmino et al., 2005), lung cancer (Takamizawa et al., 2004), Burkitt lymphoma (Metzler et al., 2004), glioblastoma (Ciafre et al., 2005), thyroid cancer (He et al., 2005), are associated with altered miRNA expression, suggesting that they may play a role in tumorigenesis.

Human pituitary adenomas are benign neoplasms, accounting for 10% of intracranial tumors. The pathogenesis of pituitary adenomas is still controversial: intrinsic pituitary defect and hormonal stimulation have been suggested as initiating events of pituitary tumorigenesis. There are evidences that support both hypotheses, indicating that the development of pituitary adenomas is a complex multistep process (Asa and Ezzat, 1998).

Pituitary adenomas can be defined as "functioning," according to the hormonal activity, giving rise to severe clinical syndromes, such as acromegaly in growth hormone (GH)-secreting pituitary adenomas, or Cushing's disease in adrenocorticotroph (ACTH)-secreting pituitary adenomas. Galactorrhea and amenorrhea are associated with prolactin (PRL)-secreting pituitary adenomas, and hyperthyroidism with thyreotroph (TSH)-secreting pituitary adenomas. On the contrary, non-functioning pituitary adenomas (NFA) do not give rise to hormone hypersecretion, but can cause symptoms of intracranial mass. Large tumors can impinge on extrasellar structures, compressing the optic chiasm and infiltrating the surrounding tissues (Asa and Ezzat, 2002).

We previously demonstrated that miR-15a and miR-16-1 are expressed at lower levels in pituitary adenomas than normal pituitary tissue, and that their expression inversely correlates with tumor diameter and directly correlates with the secretion of the anti-neoplastic cytokine p43, also called aminoacyl-tRNA synthetase-interacting multi-functional protein (AIMP1) (Bottoni

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et al., 2005). This evidence suggests that altered miRNA expression may be involved in pituitary adenomas. To verify this hypothesis and to identify specific pituitary miRNAs, we analyzed the miRNAome in 32 pituitary adenomas and in 6 normal pituitary samples by microarray containing all known human miRNAs (Liu et al., 2004).

MATERIALS AND METHODS

Human pituitary adenomas

We examined 32 pituitary adenomas, including 6 GH-secreting, 5 PRL-secreting, 4 ACTH-secreting pituitary adenomas and 17 NFA. Ten pituitary adenomas were classified as microadenomas (diameter <1 cm) and 22 as macroadenomas (diameter >1 cm). All patients with functioning pituitary adenomas and 13 patients with NFA did not receive presurgical medical treatment, while 3 NFA patients were treated with dopamine agonists before surgery. Information concerning therapy was not available in NFA patient #18. All patients underwent transsphenoidal surgery. Immunohistochemical examination for anterior pituitary hormones was performed on all specimens, confirming hormonal activity and cytodifferentiation of each tumor sample (see Supplementary table S1).

Tissue collection and RNA isolation

Tissue samples were collected in accordance with the guidelines of the local committee on human research. Tissue fragments were immediately frozen in liquid nitrogen under ribonuclease (RNase)-free conditions at the time of surgery and stored at −80°C until RNA isolation was performed. Frozen tissues were disrupted using a Dismembrator (B. Braun Biotech International, Milano, Italy) and total RNA from the pulverized tumors was isolated with TRIzol reagent (Invitrogen, Milano, Italy), and stored at −80°C until use, as previously described (Zatelli et al., 2004).

Five samples of human total RNA from normal pituitary (Analytical Biological Services, Inc., Wilmington, DE; Biochain, Hayward, CA; United States Biological, Swampscott, MA) and a pool of normal RNAs from five different individuals were used as controls.

MicroRNA microarray

RNA labeling and hybridization on miRNA microarray chips were performed as previously described (Liu et al., 2004). Briefly, 5 μg of total RNA from each sample was biotin-labeled during reverse transcription using random hexamers. Hybridization was carried out on miRNA microarray chip (ArrayExpress submitted with the following code: A-MEXP-86) (Liu et al., 2004), which contains 368 probes, including 245 human and mouse miRNA genes, in triplicate. Hybridization signals were detected by biotin binding of a Streptavidin–Alexa 647 conjugate using a Perkin-Elmer ScanArray XL5K. Scanner images were quantified by the Quantarray software (Perkin-Elmer, Wellesley, MA).

Statistical and bioinformatic analysis of microarray data

Raw data were normalized and analyzed using the GeneSpring software version 7.2 (Silicon Genetics, Redwood City, CA). Expression data were median centered both on-chip and on-gene median. Statistical comparisons were performed by using the Analysis of Variance (ANOVA) statistic and the Benjamini and Hochberg correction for false positives reduction. Cluster analysis was performed using Pearson correlation as measure of similarity.

Predictive miRNAs for pituitary adenoma histotype versus normal class were determined by using the Prediction Analysis of Microarrays software (PAM) (Tibshirani et al., 2002) (http://www-stat.stanford.edu/~tibs/PAM/index.html). The same samples were used for cross-validation and test-set prediction.

The analysis of miRNA predicted targets was done using four algorithms: TargetScan (http://genes.mit.edu/ targetscan/), PicTar (http://pictar.bio.nyu.edu/), miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), and miRBase Targets (http://microrna.sanger.ac.uk/targets/v1/). To identify the genes commonly predicted by the four different algorithms, results were intersected by using MatchMiner: (http://discover.nci.nih.gov/matchminer/MatchMinerLookup.jsp).

The analysis of experimentally supported miRNA targets was done by using Tarbase (http://www.diana.pcbi.upenn.edu/tarbase.html) (Sethupathy et al., 2005).

Reverse transcription and real-time PCR analysis

To identify specific mature miRNA levels TaqMan MicroRNA Assays have been used (P/N: 4365408 Applied Biosystems, Foster City, CA 94404), as previously described (Chen et al., 2005). Briefly, reverse transcriptase reactions included purified total RNA (2–10 ng per reaction), 50 nM stem–loop RT primer, 1 × RT buffer (P/N: 4319981, Applied Biosystems), 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe reverse trascriptase (P/N: 4319983, Applied Biosystems) and 0.25 U/μl RNase inhibitor (P/N: N8080119, Applied Biosystems). The 15 μl reactions were incubated in the Applied Biosystems 9700 Thermocycler in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate. Real-time PCR was performed using a standard TaqMan PCR kit protocol on the Applied Biosystems 7700 Sequence Detection System. The 20 μl PCR included 1.33 μl RT product, 1 × TaqMan Universal PCR Master Mix (P/N: 4324018, Applied Biosystems), as recommended by the manufacturer. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The comparative C_T method for relative quantitation of gene expression (User Bullettin #2, Applied Biosystems) was used to determine miRNA expression levels, referring all samples versus normal pool pituitary total RNA. Experiments were carried out in triplicate for each data point, and data analysis was performed by using SDS 1.7 software (Applied Biosystems). To normalize the expression levels of target genes, miR-185 has been used as reference, since preliminary experiments showed that, among the examined miRNAs, miR-185 expression levels were very similar in our samples (average expression in microarray analysis: 0.99 ± 0.11).

RESULTS

Differentially expressed and predictive miRNAs between normal pituitary and pituitary adenomas

Among all human miRNAs spotted on the chip, statistical analysis generated a list of 30 miRNAs differentially expressed between normal pituitary and pituitary adenomas (at $P < 0.05$). Seven of the genes were upregulated and 23 downregulated in pituitary adenomas. Cluster analysis, based on differentially expressed miRNAs, generated a tree showing a clear distinction between normal pituitary and pituitary adenomas (Fig. 1a) (see also Supplementary table S2).

PAM analysis identified 24 predictive miRNAs differentiating normal pituitary from pituitary adenomas. Predictive miRNAs identify a sample for being a tumor or a normal specimen on the basis of miRNA expression, with the probabilities shown in Figure 1b. Among the predictive miRNAs, we found 7 genes whose expression was increased, while the expression of 17 miRNAs was decreased as compared to normal pituitary (Table 1).

We also identified 29 miRNAs able to predict pituitary adenoma histotype (ACTH-, GH-, PRL-secreting adenomas and NFA). The histotype miRNA signature, used in test prediction, correctly identified 3 of 4 ACTH-secreting pituitary adenomas (1 is predicted as NFA), 2 out of 7 GH-secreting pituitary adenoma (see discussion, 4 of them are predicted as PRL and 1 as ACTH), 17 out of 17 NFA, and 4 out of 5 PRL-secreting adenomas (1 is predicted as NFA). Histotype predictive miRNAs are listed in Table 2 (see also a graphical representation of

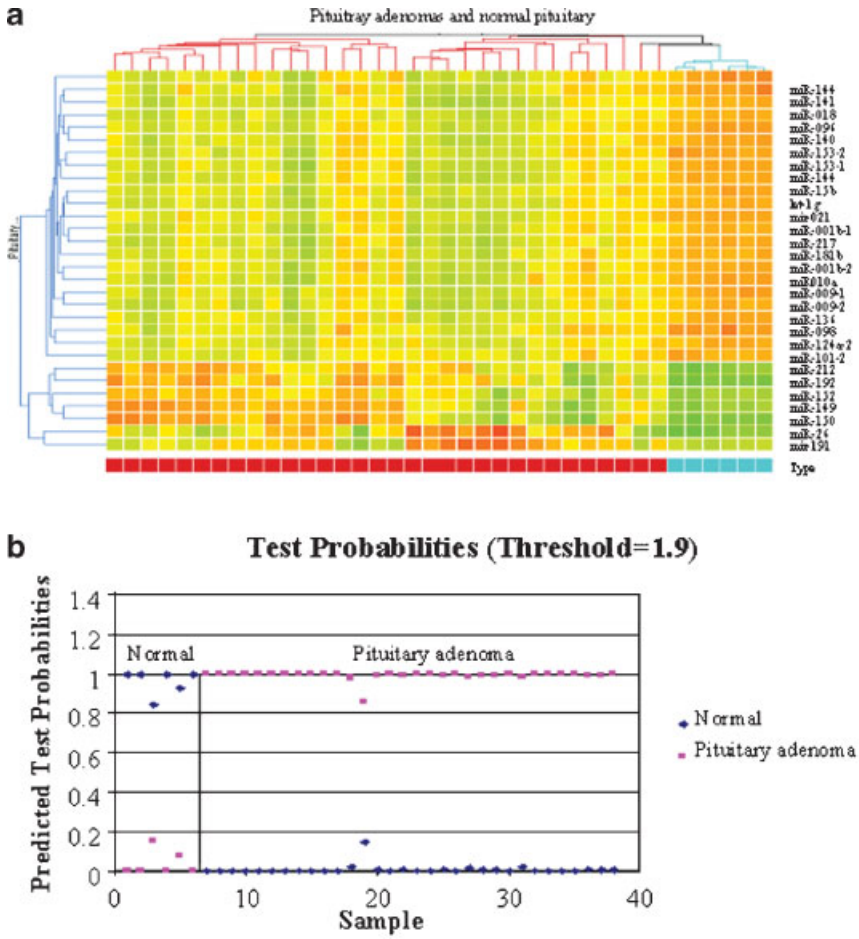


Fig. 1. Part **a**: Tree generated by cluster analysis showing the separation of pituitary adenomas from normal pituitary on the basis of miRNA differentially expressed between pituitary adenomas and normal pituitary. Part **b**: PAM analysis displaying the graphical representation of the probabilities (0.0–1.0) of each sample for being a normal pituitary sample or a pituitary adenoma.

TABLE 1. Normal pituitary and pituitary adenoma predictor miRNAs

	PAM score*	
miRNA name	Normal pituitary	Pituitary adenoma
miR-128a	1.3628	−0.2555
miR-136	1.1801	−0.2213
miR-132	0.8879	−0.1665
miR-223	0.6356	−0.1192
miR-026a	−0.45	0.0844
miR-007-3	0.3647	−0.0684
let-7a-1	0.3122	−0.0585
let-7f-1-	0.3048	−0.0572
miR-192-2/3	0.2989	−0.056
miR-026b	−0.2624	0.0492
miR-009-3	0.2192	−0.0411
miR-007-1	0.2086	−0.0391
let-7e	0.2005	−0.0376
miR-212	0.1836	−0.0344
miR-164	0.1653	−0.031
miR-138-2	0.1044	−0.0196
miR-197	−0.1044	0.0196
miR-103	−0.0819	0.0154
miR-103-2	−0.0744	0.014
miR-007-3	0.0483	−0.009
miR-192-2/3	−0.0338	0.0063
miR-149	−0.0294	0.0055
miR-100-1/2	0.007	−0.0013
miR-024-1	0.0042	0.0008

*Centroid score of the two classes of PAM.

the miRNAs listed in the table), with respective predictive scores. Figure 2 shows the probability of each sample for being predicted as a specific pituitary adenoma histotype.

Tumor diameter and miRNAs expression

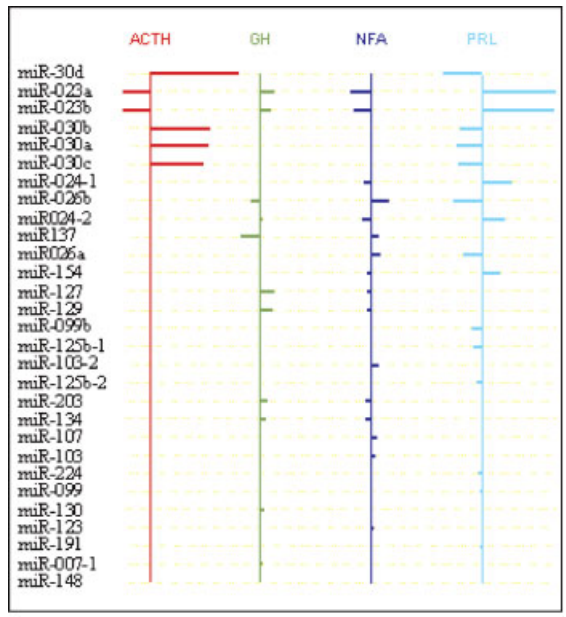
The analysis of the differentially expressed miRNAs on the basis of tumor diameter was possible only in the NFA group, which had an adequate number of samples: 4 microadenomas and 13 macroadenomas (Supplementary table S1). Six miRNAs were identified: five were upregulated and only one was downregulated in macroadenomas as compared to microadenomas (see Supplementary table S3). Figure 3a shows the cluster analysis in NFA, based on differentially expressed miRNAs between macro and microadenomas.

Pharmacological treatment and miRNAs expression

In order to investigate whether miRNAs expression is affected by pharmacological treatment, differentially expressed miRNAs were identified in NFA from patients who received pharmacological treatment (3 samples) and in non-treated NFA patients (13 samples). The analysis was not performed in functioning pituitary

TABLE 2. PAM centroid scores for miRNAs predictive of pituitary adenoma histotype

Name	ACTH score	GH score	NFA score	PRL score
miR-030a	1.3854	0	0	−0.5646
miR-030c	1.3075	0	0	−0.5137
miR-030b	1.1469	0	0	−0.475
miR-023b	0	0.0164	−0.2938	0.9632
miR-030d	0.8822	0	0	−0.5742
miR-023a	0	0.0044	−0.3229	0.7999
miR-026b	0	−0.1057	0.3131	−0.4223
miR-137	0	−0.3224	0.1141	0
miR-024-1	0	0	−0.151	0.2571
miR-154	0	0	−0.093	0.2461
miR-129-1/2	0	0.239	−0.0361	0
miR-127	0	0.2353	−0.0766	0
miR-026a	0	0	0.1769	−0.202
miR-024-2	0	0	−0.1688	0.0936
miR-103-2	0	0	0.1617	0
miR-107	0	0	0.1358	0
miR-203	0	0.0865	−0.1239	0
miR-134	0	0.0486	−0.1207	0
miR-099b	0	0	0.0349	−0.1109
miR-103-1	0	0	0.1049	0
miR-125b-1	0	0	0	−0.1026
miR-200a	0.0947	0	0	0
miR-224	0	0	0	−0.0684
miR-007-1	0	0.0675	0	0
miR-125b-2	0	0	0	−0.0546
miR-123	0	0	0.0444	0
miR-148	0	0	−0.0374	0
miR-130a	0	0.0316	0	0
miR-213	−0.0163	0	0	0



Graphical representation of predicted miRNAs listed in Tab.4

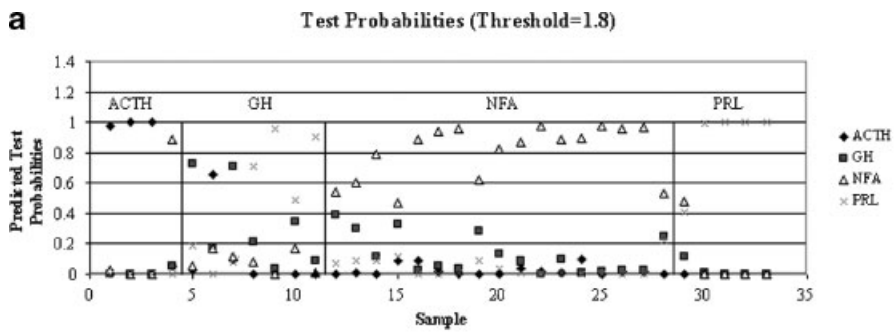
Positives and negatives scores means that the miRNA has a different expression in that class versus the other ones and is useful for the prediction.

adenomas, since none of the patients underwent medical treatment (see Supplementary table S1). As shown in Figure 3, we found six miRNAs differentially expressed: three genes were upregulated and three were down-regulated in treated NFA versus non-treated NFA. Cluster analysis, based on differentially expressed miRNA, generated a tree showing a clear distinction between pharmacologically treated and non-treated NFA (see Supplementary table S4).

Validation of differentially expressed miRNAs

To confirm the results obtained by microarray analysis, we performed Real-time PCR analysis for some of the differentially expressed miRNAs. The expression of miR-26a, miR-21, miR-141, miR-144, and miR-149 was analyzed in pituitary adenomas and in normal pituitary samples. Results obtained by Real-time PCR confirmed microarray analysis as shown in Table 3.

a



b

Test set Prediction Confusion Matrix (Threshold=1.8)				
True\Predicted	ACTH	GH	NFA	PRL
ACTH	**3**	0	1	0
GH	1	**2**	0	4
NFA	0	0	**17**	0
PRL	0	0	1	**4**

Fig. 2. Part **a**: PAM analysis displaying the probability of each sample to be one of the four pituitary adenoma histotypes (ACTH, GH, NFA, PRL). Part **b**: Table showing the Test set Prediction Confusion Matrix, indicating the samples correctly identified as belonging to the different pituitary adenoma histotypes.

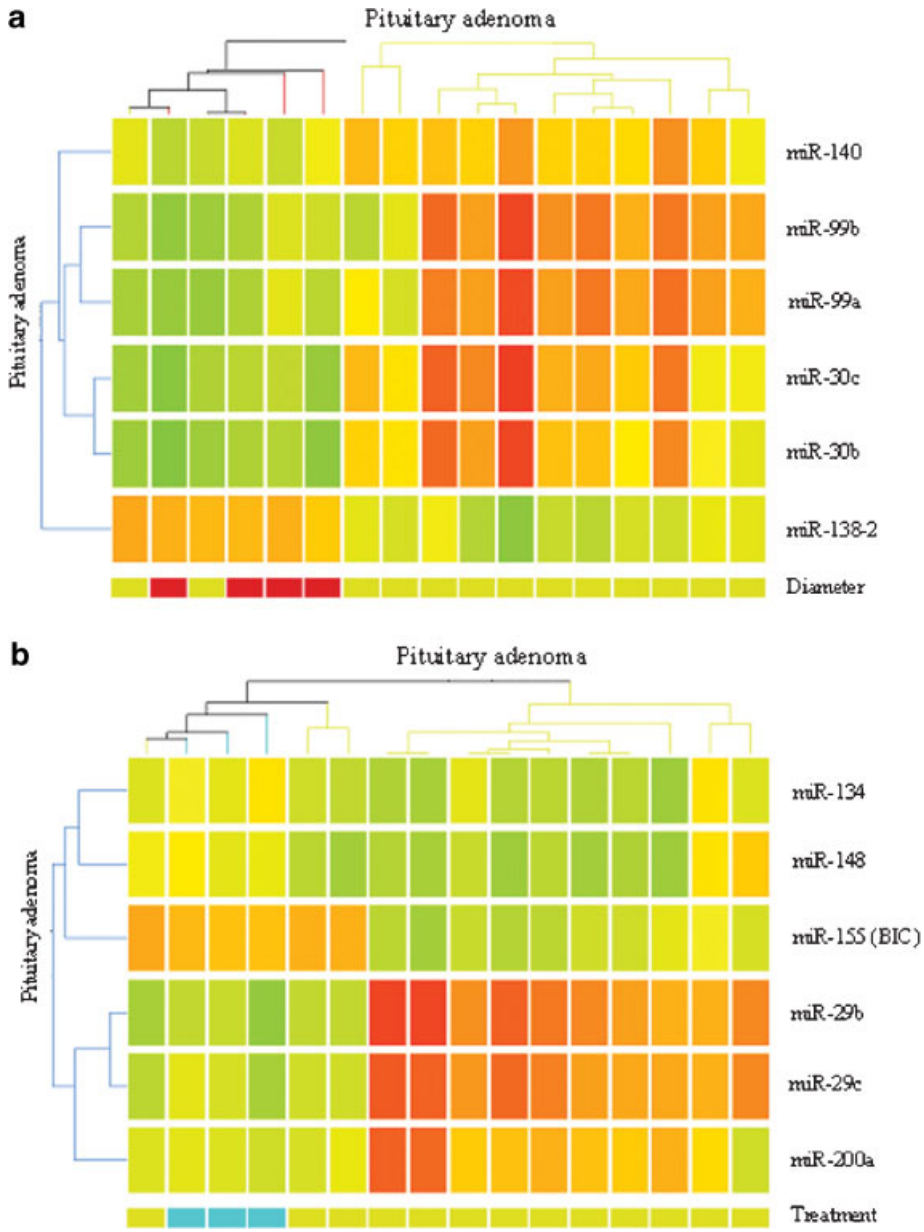


Fig. 3. Part **a**: Tree generated by cluster analysis showing the separation of NFA in micro- and macro-adenomas on the basis of differentially expressed miRNAs. Part **b**: Tree generated by cluster analysis showing the separation of NFA in pharmacologically and not pharmacolgically treated on the basis of differentially expressed miRNAs.

TABLE 3. Microarray data validation by real-time PCR

	miRNA-26a		miRNA-21		miRNA-141		miRNA-144		miRNA-149	
	Microarray	Real time	Microarray	Real time	Microarray	Real time	Microarray	Real time	Microarray	Real time
Normal pool	1	1	1	1	1	1	1	1	1	1
1	4.14	3.20	0.48	0.16	0.46	0.50	0.45	0.40	2.17	2.10
2	2.23	3.30	0.57	0.42	0.47	0.52	0.47	0.45	2.92	2.10
3	8.35	8.00	0.50	0.57	0.46	0.60	0.47	0.60	1.54	7.57
4	3.44	3.80	0.49	0.45	0.47	0.33	0.47	0.20	1.96	9.30
5	5.17	3.50	0.57	0.39	0.54	0.53	0.52	0.43	2.08	2.00
6	2.48	2.20	0.57	0.89	0.54	0.60	0.58	0.20	2.17	1.80

Real-time PCR was performed for miR-26a, miR-21, miR-141, miR-144, and miR-149. The values indicate fold induction (FI) of miRNA expression levels versus normal pool for each analysed samples. Relative miRNA mean levels in pituitary adenomas were evaluated by Real-time PCR in three individual experiments.

Target analysis

The analysis of predicted target genes was performed for the most differentially expressed miRNAs in pituitary adenomas versus normal pituitary (miR-024-1, miR-212, miR-026a, miR-098), by using the algorithms described in the Materials and Methods section. Results are shown in Supplementary table S5. We also analyzed the experimentally supported microRNA targets for miR-001, miR-15, miR-16, miR-24, miR-26a, miR-30a, miR-101, miR-124, miR-125b, miR-127, miR-130, miR-132, miR-136, and miR-141. Results are shown in Supplementary table S6.

DISCUSSION

To explore the miRNAome in pituitary adenomas and in normal pituitary gland, we analyzed 32 pituitary adenomas and 6 normal pituitaries using a miRNA microarray. Thirty miRNAs were identified as differentially expressed between pituitary adenomas and normal pituitary, suggesting that miRNAs genes may be involved in pituitary adenoma development. Among the differentially expressed miRNAs, the most representative were miR-212, miR-026a, miR-150, miR-152, miR-191, and miR-192, which were upregulated in pituitary adenomas, while miR-024-1 and miR-098 were downregulated in tumor samples. Some of the upregulated miRNAs in pituitary adenomas were described to be involved in cell growth and apoptosis (Cheng et al., 2005). Cheng et al. (2005) inhibited the expression of many miRNAs, including miR-150, miR-152, miR-191, and miR-192 (upregulated in pituitary adenomas), with consequent decrease in cell growth. Our results, therefore, suggest that the overexpression of these miRNAs might result in an increased pituitary cell proliferation, indicating that altered expression of miRNA genes may be important in the development of pituitary tumors.

We identified 24 miRNAs able to correctly predict the nature of each sample (adenoma or normal pituitary). Among miRNAs predictive of pituitary adenomas, we found that miR-26a and miR-26b expression was increased as compared to its expression in the normal samples. miR-26a is probably involved in neural cell specification, with a stronger expression in astrocytes (Smirnova et al., 2005). On the contrary, miR-132, miR-128a, miR-136, miR-16-1, and let-7 expression was decreased as compared to their expression in normal pituitary. miR-132 was described to be involved in tumor growth (Cheng et al., 2005), while the expression of miR-128a was downregulated also in human glioblastoma (Ciafre et al., 2005). It is of interest to notice that miR-128a is a brain-enriched miRNA, activated during neuronal differentiation (Sempere et al., 2004). Among the predictive miRNAs, we confirmed miR-16-1, whose expression was lower in pituitary adenomas as compared to normal pituitary tissue, in agreement with our previous findings (Bottoni et al., 2005). Taken together, these findings support the hypothesis that miR-16-1 may be involved in pituitary tumor growth. The possible role of miR-16-1 in tumors is supported by the evidence that its expression is downregulated in the majority of B-CLL cases (Calin et al., 2002). Moreover, one of miR-16-1 target was shown to be BCL2, which is upregulated possibly as a consequence of miR-16-1 downregulation in human B-CLL, thus protecting cells from apoptosis (Cimmino et al., 2005). It was also found that BCL2 oncoprotein is expressed at high level in approximately one-third of pituitary adenomas, while no immunoreactivity was detected in normal pituitary (Wang et al., 1996), suggesting that BCL2 plays a role in the regulation of apoptotic mechanisms in pituitary adenomas too.

In accordance with previous results, which demonstrated let-7 downregulation in breast (Iorio et al., 2005) and in lung cancer (Takamizawa et al., 2004), let-7 expression was also reduced in pituitary adenomas, possibly causing an upregulation of members of the human RAS family of oncoproteins.

Microarray analysis identified miRNAs whose expression could differentiate the four histotypes of pituitary adenoma. We found that GH-secreting and PRL-secreting adenomas share a common signature: miR-23a, miR-23b, and miR-24-2 expression was increased in these samples. In particular, miR-23a and miR-23b increased expression differentiates GH-secreting and PRL-secreting adenomas from ACTH-secreting adenomas and NFA, where the expression of these miRNAs is decreased. As recently described by Cheng et al. (2005), the inhibition of miR-24 and miR-23 caused a decrease of cell growth in lung carcinoma cells. Our results, therefore, suggest that these two miRNAs may be involved in the development of pituitary tumors. Anyway, the functions of miR-23 and miR-24 are still unknown, and further studies are required to clarify the role of these genes in cellular biology.

miR-26b expression was lower in GH-secreting and PRL-secreting adenomas as compared to NFA, differentiating this last group of tumors from the other two histotypes. The common signature shared by PRL-secreting and GH-secreting adenomas is consistent with the common origin of GH- and PRL-secreting pituitary cells from the somatotroph stem cells (Asa and Ezzat, 1998). Moreover, this evidence possibly explains why the PAM analysis predicted four GH-secreting adenoma samples as PRL-secreting adenomas. Furthermore, an immunohistochemical analysis performed on 69 acromegalic tumors revealed PRL-immunopositive cells in 90% of somatotroph tumors (Ezzat et al., 1995) suggesting a further explanation for the incorrect prediction of the analyzed samples. On the other hand, immunohystochemical analysis showed the lack of GH staining in the examined PRL-secreting adenomas, in agreement with the fact that none of the PRL-secreting adenomas was predicted by the PAM analysis as a GH-secreting pituitary adenoma.

miRNA expression profile in ACTH-secreting adenomas is clearly different from other histotypes. miR-30a, miR-30b, miR-30c, and miR-30d expression was strongly increased in this group, differentiating this class of adenomas from PRL-secreting adenoma histotype, where the expression of these miRNAs is lower. Also in this case, we speculate that this unique profile of miRNA expression could be ascribed to the early determination of corticotroph lineage during pituitary cytodifferentiation (Asa and Ezzat, 1998).

Furthermore, also NFA display an unique miRNA expression profile. Indeed, we found that miRNA-24-2 expression was decreased in this histotype, differentiating these tumors from GH-secreting and PRL-secreting adenomas, where the expression of this miRNA was increased.

miR-26a expression distinguished NFA from PRL-secreting adenomas, as the expression of this miRNA was increased in the first group as compared to the second histotype. The expression of miR-127, miR-129, miR-203, and miR-134 was reduced in NFA as compared to GH-secreting adenomas, differentiating these two

classes of tumors. Finally, miR-137 expression was higher in NFA than in GH-secreting adenomas, allowing distinction of these two histotypes. Despite NFA are considered heterogeneous tumor entities, their miRNA expression profile could differentiate this histotype from other pituitary adenomas except from ACTH-secreting adenomas. Our results, therefore, suggest that miRNAs could be useful diagnostic markers, improving the classification of pituitary adenoma histotypes. Moreover, discovering the targets of these predictive miRNAs might shed some light on cytodifferentiation processes.

Among the six miRNAs differentially expressed between micro and macro NFA, miR-140 was upregulated in macroadenomas. It has been shown that inhibition of miR-140 expression reduces cell growth in the lung carcinoma cell line A549 (Cheng et al., 2005), suggesting that an increased expression of this miRNA in NFA could be involved in the control of tumor growth. As we can see in the cluster analysis, two macroadenomas are grouped with microadenomas, suggesting that the diameter of these samples might be more similar to that of microadenomas. However, the only information available is that sample diameters were greater than 1 cm, since precise information concerning the measure was lacking, and therefore, we cannot confirm this assertion.

We then evaluated whether pharmacological treatment could affect miRNAs expression in NFA patients treated or not with dopamine agonists before surgery. Among the six differentially expressed miRNA, miR-148 was upregulated in treated samples. It has been demonstrated that miR-148 expression inhibition increases the level of apoptosis in HeLa cells (Cheng et al., 2005). This finding is in contrast with our data, since it was described that the anti-tumoral action of bromocriptine is connected with the induction of apoptosis (Gruszka et al., 2004). However, Cheng et al. (2005) found opposite effects of the same miRNA in two cell lines, suggesting that the targets of the miRNA may be different, or that miRNA targets may have different activities. In any case, it is notable that pharmacological treatment may affect miRNA expression, indicating that they might represent an important target for pharmacological treatment.

The lack of knowledge about miRNA target genes hampers the full understanding on the biological functions of miRNAs. We, therefore, analyzed the predicted miRNA target genes for two downregulated and two upregulated miRNAs in pituitary adenomas. Among putative targets of miR-24, which expression is downregulated in pituitary adenomas, we found Caudal-type homeobox protein 2 (CDX-2), vascular endothelial growth factor receptor 1 (VEGFR-1), human protooncogene proviral insertion site in moloney murine leukemia virus kinase (Pim-1), and the guanine nucleotide exchange factor Vav-1. CDX-2 is a transcription factor, expressed at high levels in 81% of intestinal neuroendocrine carcinomas (Barbareschi et al., 2004). VEGFR-1 is correlated with different cancers (Luttun et al., 2004) and has been demonstrated to be upregulated in NFA (McCabe et al., 2002). Pim-1 kinase is an oncogene, involved in the control of cell growth, differentiation, and apoptosis, and its overexpression is a potential biomarker in prostate carcinoma (Xu et al., 2005). Finally, the Vav family of proto-oncogenes has been implicated in the regulation of numerous pathways downstream of receptor tyrosine kinases participating in tumorigenesis (Billadeau, 2002). Accordingly, one of the predicted target genes for miR-98, which expression is downregulated in pituitary adenomas, is RIO kinase 3, which belongs to a family of kinases required for proper cell-cycle progression and chromosome maintenance (Laronde-Leblanc and Wlodawer, 2005). Other predicted target genes are involved in cytoskeleton organization and in vesicle trafficking, such as CK2 interacting protein 1 (Olsten et al., 2004) and Syntaxin-17 (Sorensen, 2005). These reports confirm that many putative targets for downregulated miRNAs, such as miR-24 and miR-98, encode proteins involved in cell growth and proliferation or with potential oncogenic functions.

On the contrary, miR-26a and miR-212 expression is strongly upregulated in pituitary adenomas. Among the predicted targets for miR-26a, we found homeobox protein Hox-A5, which expression is reduced or absent in active angiogenic endothelial cells found in association with breast tumors or in proliferating infantile hemangiomas (Rhoads et al., 2005). Moreover, Hox A5 was recently shown to suppress growth and induce p53-dependent apoptosis, and its expression was higher in differentiated compared to undifferentiated colon epithelial cells (Wang et al., 2001).

Among miR-212 putative targets, we found Death effector domain-containing protein (DEDD), a protein involved in apoptotic signaling (Stegh et al., 1998), as well as other proteins participating to apoptosis. Further studies are required to determine the actual miRNA targets in order to highlight the role of these genes in disease and cancer.

We also analyzed the experimentally supported targets of some microRNA. Among the validated targets we found transcription factors, oncogenes, genes involved in histone deacetylation and in cancer cell invasion, metastasis, and angiogenesis. Among these genes, pleomorphic adenoma gene 1 (*PLAG1*), described as a target of miR-26a (Volinia et al., 2006), is a proto-oncogene involved in the pathogenesis of many human tumors. *PLAG1* is a member of the PLAG family of tumor genes, including also *ZAC1*. The latter gene was shown to be highly expressed in normal anterior pituitary gland, but downregulated in most pituitary adenomas (Pagotto et al., 2000), suggesting that this gene family might be involved in the development of pituitary adenomas.

However, none of the predicted or validated target genes, except for *BCL2*, has been analyzed in normal and pathologic pituitary, so far, leaving the research field wide open.

In conclusion, our results present an extensive miRNAs expression analysis in pituitary adenomas and in normal pituitary gland, which allows the identification of differentially expressed miRNAs between normal gland and pituitary adenomas. Moreover, our data revealed a miRNA signature predicting the nature of the sample analyzed (normal vs. neoplastic), and a specific signature differentiating pituitary adenoma histotypes. Furthermore, we show that in NFA a group of miRNAs associates with tumor diameter and another group with pharmacological treatment.

Several miRNAs identified in our study are involved in cell proliferation and apoptosis, indicating that deregulated miRNA expression may be involved in pituitary neoplastic transformation. Moreover, predictive miRNAs could be useful as diagnostic markers, improving the classification of pituitary adenoma histotypes. Studying the target of deregulated miRNAs genes may elucidate biological functions involved in pituitary adenoma pathogenesis.

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