MicroRNA Signature of Primary Pigmented Nodular Adrenocortical Disease: Clinical Correlations and Regulation of *Wnt* Signaling

Dimitrios Iliopoulos,[1] Eirini I. Bimpaki,[2] Maria Nesterova,[2] and Constantine A. Stratakis[2]

[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts and [2]Section on Endocrinology and Genetics, Program in Developmental Endocrinology and Genetics, National Institute of Child Health and Human Development, NIH, Bethesda, Maryland

Abstract

MicroRNAs comprise a novel group of gene regulators implicated in the development of different types of cancer; however, their role in primary pigmented nodular adrenocortical disease (PPNAD) has not been investigated. PPNAD is a bilateral adrenal hyperplasia often associated with Carney complex, a multiple neoplasia syndrome; both disorders are caused by protein kinase A (PKA) regulatory subunit type 1A (*PRKAR1A*)–inactivating mutations. We identified a 44-microRNA gene signature of PPNAD after comparing PPNAD with normal adrenal samples. Specifically, 33 microRNAs were up-regulated and 11 down-regulated in PPNAD relative to normal tissues. These results were validated by stem loop real-time PCR analysis. Comparison of microRNA microarray data with clinicopathologic variables revealed a negative correlation ($r = -0.9499$) between *let-7b* expression and cortisol levels in patients with PPNAD. Integration of microRNA microarray with serial analysis of gene expression data together with bioinformatic algorithm predictions revealed nine microRNA-gene target pairs with a potential role in adrenal pathogenesis. Using a PPNAD cell line, we showed that miR-449 was up-regulated and identified its direct target, WNT1-inducible signaling pathway protein 2 (*WISP2*); in addition, pharmacologic inhibition of PKA resulted in the up-regulation of miR-449 leading to the suppression of *WISP2*. Overall, we investigated, for the first time, the microRNA profile and its clinical significance in PPNAD; these data also suggest that PKA, via microRNA regulation, affects the Wnt signaling pathway, which through expression and clinical studies is suspected to be a primary mediator of *PRKAR1A*-related tumorigenesis. [Cancer Res 2009;69(8):3278–82]

Introduction

Primary pigmented nodular adrenocortical disease (PPNAD) is a rare form of bilateral adrenal hyperplasia that is linked to Carney complex (1). PPNAD is the most frequent endocrine tumor in patients with Carney complex and it presents with excess cortisol secretion and symptoms of Cushing syndrome in early life (2). PPNAD is caused by inactivating mutations of the gene coding for the regulatory (R) subunit 1A of the protein kinase A (PKA) enzyme (*PRKAR1A*). According to biochemical studies, PPNAD tumors exhibit increased PKA activity upon cyclic AMP stimulation (3), mainly attributed to the overexpression of other regulatory subunits, deficient control of the catalytic subunit, and consequently, deregulation of the PKA holoenzyme (4). A recent study revealed the differential expression of several important genes implicated in steroidogenesis and cell proliferation signaling pathways between PPNAD and normal adrenal tissues (5).

MicroRNAs consist of a novel class of gene regulators, specifically in the posttranscriptional level (6). MicroRNA expression has been studied extensively in the past few years, establishing microRNA signatures in several types of cancer (7). In the present study, we identified a microRNA gene signature for PPNAD; furthermore, we found that *let-7b* microRNA was highly associated with midnight cortisol levels, an index of clinical severity of the Cushing syndrome caused by PPNAD tumors. In addition, using a PPNAD cell line, we identified the inhibition of miR-449 and up-regulation of its target gene WNT1-inducible signaling pathway protein 2 (*WISP2*). These results suggest that PKA, through microRNA regulation, affects the Wnt signaling pathway, which several other studies have shown is important in the regulation of *PRKAR1A*-related tumorigenesis and in adrenocortical oncogenesis in general.

Materials and Methods

Subjects. Patients were seen at the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development under protocol 95-CH-0059, approved by the Institutional Review Board. All patients gave their informed consent and signed the related forms. We studied 10 patients diagnosed with Cushing syndrome caused by Carney complex and/or PPNAD (Supplementary Table S1; Supplementary Methods). Adrenal samples were collected during bilateral adrenalectomy, dissected and kept in −80°C until use. Four normal adrenal samples were used as controls; three of them were commercially available RNA adrenal samples (Ambion, Biochain) and a single normal adrenal tissue sample obtained from a cadaver through the National Development and Research Institutes, Inc. (New York, NY).

MicroRNA expression analysis. The RNA isolation was performed using the mirVana microRNA isolation Kit (Ambion) according to the manufacturer's instructions. TaqMan microRNA array assays were used in order to study the expression levels of 365 microRNAs as previously described (8).

PPNAD cell line. We used a cell line that was derived from the adrenocortical tissue of patient CAR 47.01 (Supplementary Table S1), as previously described (ref. 9; Supplementary Methods).

H89 treatment. PPNAD cells were treated with 1 μmol/L of the PKA inhibitor H89; miR-449 expression was then evaluated by real-time PCR analysis. WISP2 mRNA expression was evaluated 24 h after H89 treatment by real-time PCR. Real-time PCR experiments were performed in triplicate and the data are presented as mean ± SD.

Statistical analysis. A two-sample *t* test was done for experiments described above. Experiments were done at least in triplicate, and a mean was calculated. $P < 0.05$ was considered significant.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

D. Iliopoulos and E.I. Bimpaki contributed equally to this work.

Requests for reprints: Constantine A. Stratakis, Section on Endocrinology and Genetics/Program in Developmental Endocrinology and Genetics, National Institute of Child Health and Human Development, NIH, Building 10, CRC (East Laboratories), Room 1-3330, 10 Center Drive, MSC1103, Bethesda, MD 20892. Phone: 301-496-4686, ext. 496-6683; Fax: 301-402-0574, ext. 480-0378; E-mail: stratakc@mail.nih.gov.

doi:10.1158/0008-5472.CAN-09-0155

Results

MicroRNA gene signature in PPNAD. We studied the expression of 365 microRNAs in 10 adrenal samples that we collected from patients with PPNAD and compared them with four normal adrenal samples. We detected 44 differentially expressed microRNAs between PPNADs and normal samples. Specifically, 33 microRNAs were down-regulated, whereas 11 were up-regulated in PPNADs in comparison to normal samples (Fig. 1). Real-time PCR analysis paralleled our microRNA array results and confirmed the differential expression of the 44 microRNAs in PPNAD compared with normal adrenal samples (Supplementary Fig. S1). Among the highly down-regulated microRNAs, we detected miR-200c (−10.74-fold), miR-200b (−9.57-fold), miR-375 (−9.97-fold), and miR-449 (−8.42-fold). On the other hand, miR-594 (7.38-fold) and miR-301 (7.57-fold) were the most highly up-regulated ones; the other microRNAs with increased expression had an increase of 1.84-fold to 3.94-fold.

***Let-7b* expression correlated with severity of Cushing syndrome due to PPNAD.** We classified our patients on the basis of their midnight cortisol levels into three groups (Supplementary Table S1). The first group included the patients with midnight cortisol levels <10 μg/dL (patients 4, 6–8), the second had cortisol levels between 10 and 20 μg/dL (patients 1–3), and the third group included patients with cortisol levels >20 μg/dL (patients 5, 9, and 10). Comparison between microRNA expression and midnight cortisol levels revealed a significant inverse correlation between *let-7b* expression and midnight cortisol levels ($r = -0.9499$; Supplementary Fig. S2).

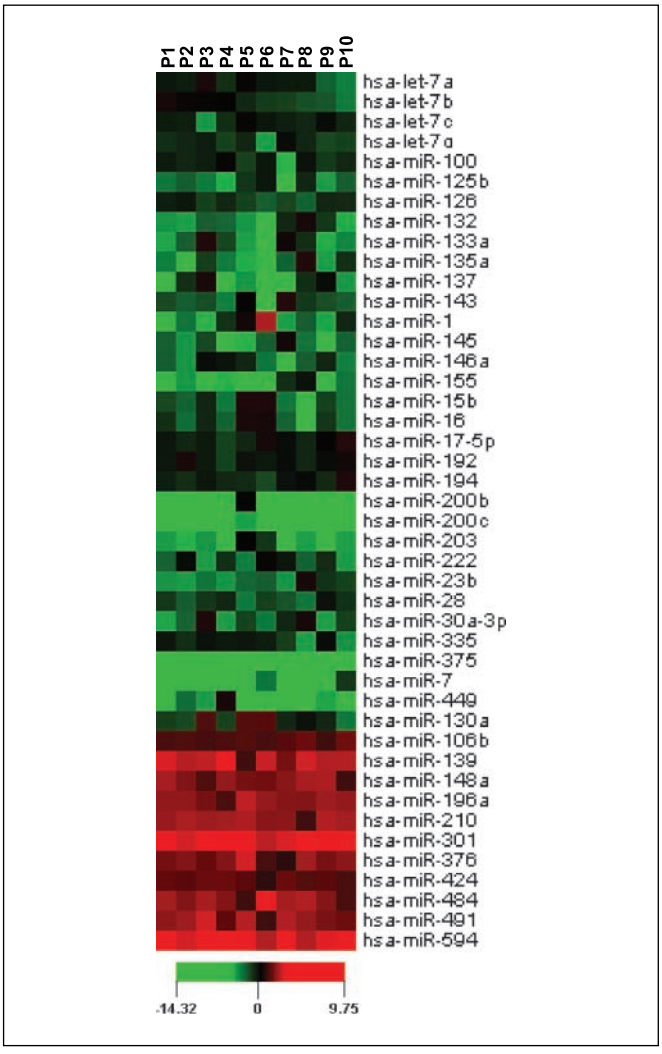

Figure 1. PPNAD microRNA gene signature. Heat map representation of 44 differentially expressed microRNAs between 10 PPNAD and 4 normal adrenal tissues. A color map is used to visualize the difference in expression; *green,* low expression; *red,* high expression. Thirty-three microRNAs were down-regulated, whereas 11 were up-regulated in PPNAD relative to normal adrenal tissues.

Integration of microRNA with serial analysis of gene expression data together with bioinformatic algorithms predicts microRNA-gene target pairs in PPNAD. Because microRNA exert their biological functions through the suppression of target genes, it is important to identify microRNA-gene target pairs. Recently, Huang and colleagues used microRNA with cDNA expression profiling data to identify human microRNA targets (10). To identify statistically significant microRNA gene targets, we selected only the microRNA gene targets that were predicted by three different bioinformatic algorithms (Supplementary Fig. S3; Supplementary Methods). We then integrated our microRNA and serial analysis of gene expression data (5) and selected only the microRNA-gene target pairs that were expressed in patients with PPNAD and were inversely correlated.

We identified nine microRNA-gene target pairs that are potentially implicated in PPNAD pathogenesis (Table 1; Supplementary Fig. S4). Specifically, miR-203 potentially targets murine osteosarcoma viral oncogene homologue B (*FOSB*), whereas miR-7 potentially targets inhibin-α (*INHA*). Both miR-15 and miR-16 potentially target serine/threonine kinase 19 (*STK19*) and cyclin D2 (*CCND2*); also, miR-16 potentially regulates chromogranin A (*CHGA*) expression. Additionally, miR-7 potentially regulates G protein–coupled receptor 107 (*GPR107*) and miR-449 is predicted to regulate *WISP2*. Real-time PCR analysis of these nine microRNA-gene target pairs revealed that the miR-449–WISP2 pair was the highest inversely correlated in our PPNAD tissues ($r = -0.9743$; Supplementary Figs. S5 and S6).

MiR-449 regulates WISP2 expression in PPNAD cellular system. Due to the fact that miR-449 was one of the highest down-regulated microRNAs in PPNAD and is predicted to regulate WISP2, an important player of Wnt signaling pathway, we further studied the role of miR-449–*WISP2* interaction in PPNAD pathogenesis using a cell line derived from a patient with PPNAD. In these PPNAD cells, we identified that *WISP2* was highly expressed (3.6-fold) in comparison to normal adrenal samples (Fig. 2*A*). On the other hand, miR-449 expression was down-regulated (3.84-fold) in the PPNAD cell line (Fig. 2*A*). The inverse correlation between miR-449 and WISP2 mRNA expression levels suggested that miR-449 regulates the Wnt pathway in PPNAD. According to our integrative analysis, we found that miR-449 directly targets *WISP2* (Table 1; Supplementary Fig. S4). MiR-449 overexpression (50 nmol/L) suppressed >70% *WISP2* mRNA expression (Fig. 2*B*). To identify if miR-449 directly regulated *WISP2* through binding in the 3′-untranslated region (UTR), we performed luciferase assay which revealed that miR-449 inhibited >60% *WISP2* 3′-UTR luciferase activity, suggesting that miR-449 directly regulates *WISP2* expression (Fig. 2*C*).

PKA activates the Wnt pathway through miR-449 inhibition. In order to detect if miR-449 is regulated by PKA, we treated PPNAD cells with high PKA activity (9) with H89 (1 μmol/L) and tested miR-449 expression. Inhibition of PKA activity by H89 increased miR-449 expression (Fig. 3*A*), followed by a

Table 1. Predicted microRNA-gene target pairs in PPNAD

MicroRNA gene	Chromosomal location*	Putative targets †	Description
miR-203	14: 103653495-103653604	*FOSB*	FBJ murine osteosarcoma viral oncogene homologue B
miR-7	9: 85774483-85774592	*INHA*	Inhibin-α
miR-15b	3: 161605070-161605167	*STK19*	Serine/threonine kinase 19
		CCND2	Cyclin D2
		CHGA	Chromogranin A (parathyroid secretory protein 1)
miR-16	3: 161605227-161605307	*STK19*	Serine/threonine kinase 19
		CCND2	Cyclin D2
miR-28	3: 189889263-189889348	*GPR107*	G protein–coupled receptor 107
miR-449	5: 54502117-54502207	*WISP2*	WNT1 inducible signaling pathway protein 2

*MicroRNA chromosomal location (mouse genome) according to the miRBase database from Sanger Institute.
†Putative microRNA targets fulfilling the criteria described in Supplementary Figure 3.

decrease in *WISP2* expression (Fig. 3*B*). To further investigate if PKA affects WISP2 expression through miR-499, we transfected the same PPNAD cells with increasing concentrations of the inhibitor of miR-449 (as-miR-449) together with H89 (Fig. 3*C*). H89, which up-regulated miR-449 expression, down-regulated *WISP2* mRNA by >60% (*lane 2*), whereas treatment with as-miR-499 blocked the inhibitory effect of H89 (*lanes 4–6*). These data suggested that PKA activation blocks miR-449 expression and

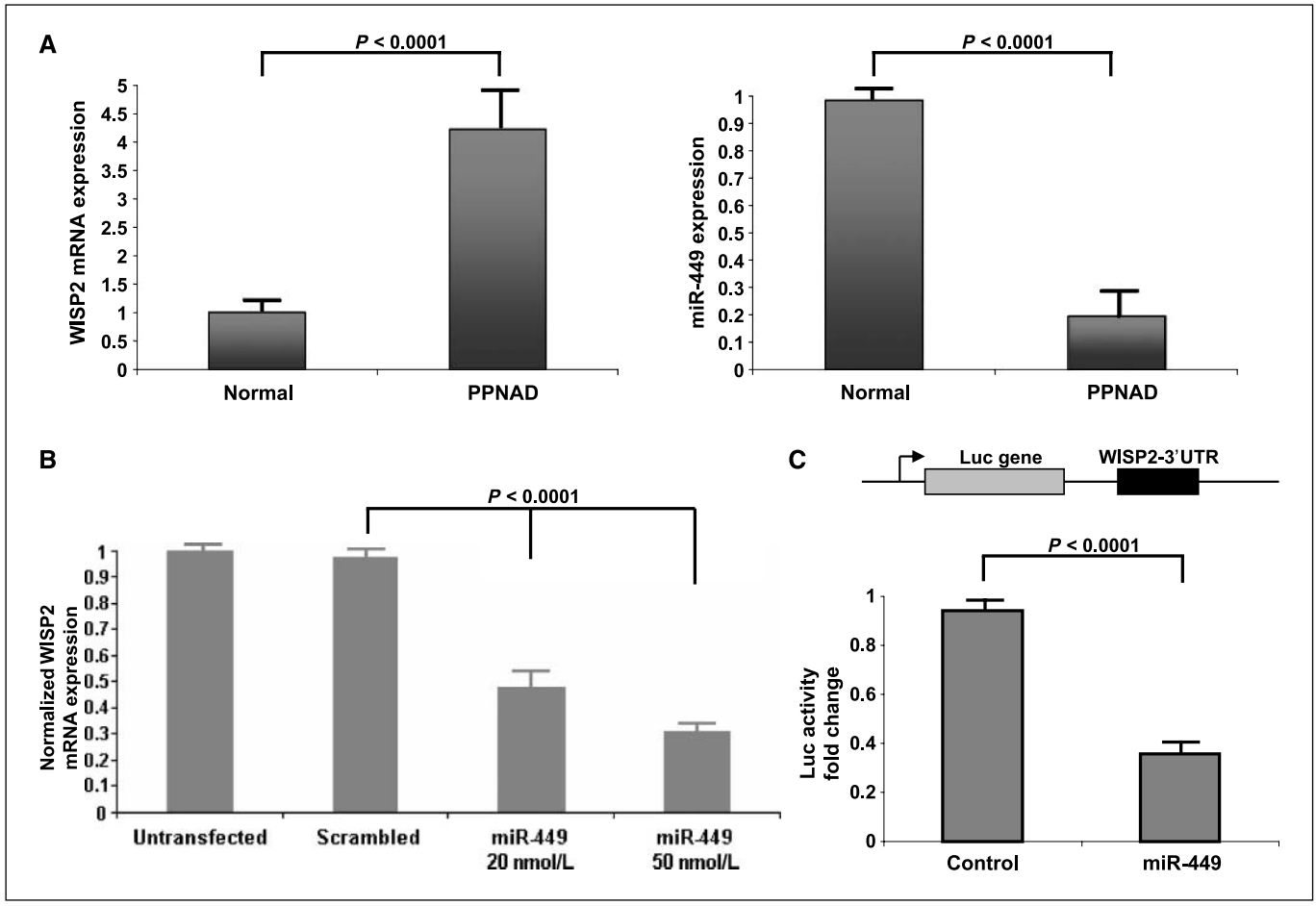

Figure 2. miR-449 directly regulates WISP2 expression. *A,* real-time PCR analysis of *WISP2* and miR-449 expression levels in a PPNAD cell line. *WISP2* is overexpressed whereas miR-449 is down-regulated in PPNAD cells relative to normal adrenal tissue. *B,* transfection of miR-449 in our PPNAD cell line (20 and 50 nmol/L) reduced *WISP2* expression. *C,* luciferase assay after transfection of miR-449 (50 nmol/L) together with luciferase vector harboring the 3′-UTR of *WISP2* gene. MiR-449 inhibited >60% of *WISP2* 3′-UTR luciferase activity.

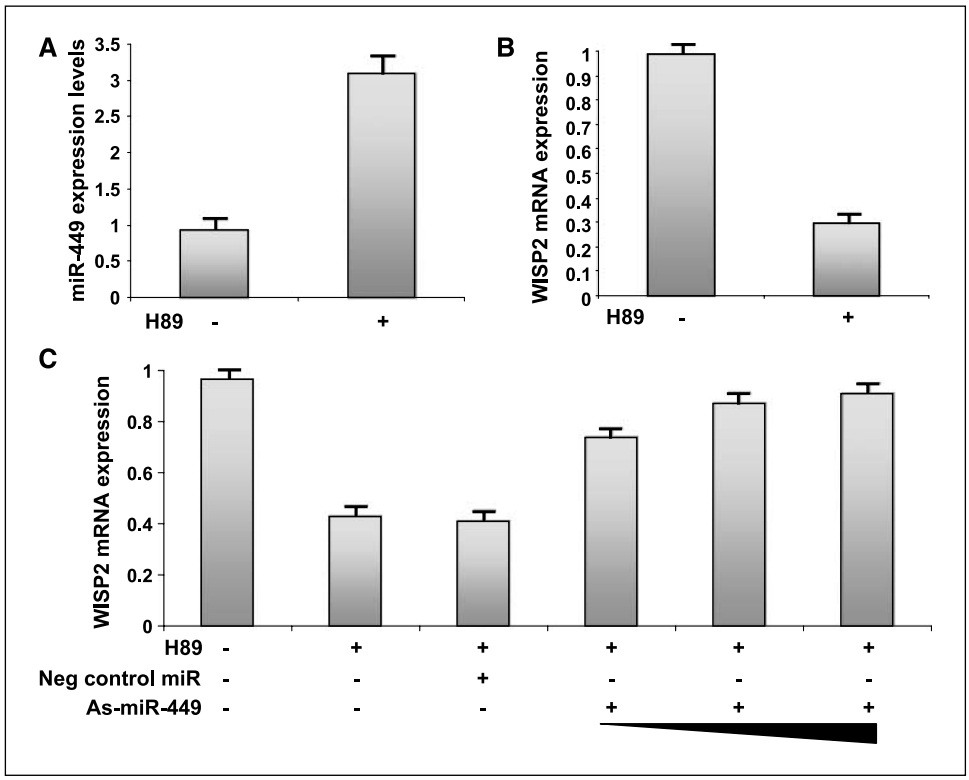

Figure 3. PKA affects *WISP2* expression through miR-449 regulation. Inhibition of PKA activity using PKA inhibitor H89 (at 1 μmol/L) increased (*A*) miR-449 expression and decreased (*B*) *WISP2* mRNA expression. *C,* real-time PCR analysis of *WISP2* expression after H89 treatment together with increasing concentrations of as-miR-449 (20, 50, and 100 nmol/L). Transfection with negative control (100 nmol/L) microRNA was used as a transfection control.

miR-449 inhibition allows the up-regulation of its target gene *WISP2*.

To test if PKA regulates miR-449 and WISP2 expression only in PPNAD cells, we treated Saos-2 osteosarcoma cells with forskolin and identified a >70% down-regulation of miR-449 expression whereas WISP2 mRNA expression levels were increased (5.6-fold; Supplementary Fig. S7; Supplementary Methods). Furthermore, H89 treatment blocked the effect of forskolin in miR-449 and WISP2 expression levels (Supplementary Fig. S7). Overall, these data suggest that PKA regulates miR-449 and WISP2 expression not only in PPNAD cells but also in other cancer cells.

Discussion

This is the first study of microRNAs in PPNAD and, indeed, in any form of adrenal hyperplasia. We detected 44 microRNAs which were differentially expressed in PPNAD versus normal adrenal tissue. The PPNAD microRNA signature revealed an aggressive phenotype that is similar to the microRNA profiles of various malignancies: it is characterized by significant down-regulation of microRNAs (33/44). According to previous reports, in carcinogenesis, there is mainly suppression of microRNA expression (7). Several of the microRNAs identified to be down-regulated in PPNAD relative to normal adrenal tissues have been implicated in tumorigenesis. Specifically, four members of the let-7 family (*let-7a*, *let-7b*, *let-7c*, and *let-7g*) were down-regulated in PPNADs, suggesting that the aggressiveness and increased cell proliferation of PPNADs may be also due to *let-7* down-regulation. The *let-7* family is a very important microRNA family in cancer which seems to have a tumor suppressor role through different mechanisms (11). Furthermore, miR-200b and miR-200c, both highly down-regulated microRNAs in PPNAD tissues, are important regulators in epithelial-mesenchymal transition (12). We also identified miR-449 as one of the highest down-regulated microRNAs in PPNAD; its function has not been studied previously. Additionally, we detected microRNAs with increased expression in PPNAD tissues. Among them, miR-210 has been found up-regulated in breast cancer and is regulated by the hypoxia-inducible factor (*HIF1A*; ref. 13).

To discover a putative association between microRNA expression levels and the severity of the disease, we studied the correlation of different clinicopathologic variables such as diurnal cortisol levels, urinary-free cortisol levels, and 17-OH-steroids with the PPNAD microRNA signature. All these variables are important diagnostic factors of PPNAD and previous studies have shown that midnight cortisol levels correlate with disease severity (14). In addition, it has been suggested that cortisol oversecretion in adrenocortical carcinomas is related to poor prognosis (2). *Let-7b* was found to be negatively correlated to these clinical indices.

Several bioinformatic algorithms have been constructed in order to predict microRNA gene targets. These algorithms predict hundreds of potential gene targets, which cannot all be experimentally validated. To clarify the biological effects of microRNAs in PPNAD, we identified their putative gene targets using the available bioinformatic algorithms and we integrated the computationally predicted target genes with expression data on PPNAD that we have previously published (5). Interestingly, the most significant correlation was found between miR-449 and *WISP2* expression levels, supporting the potential involvement of Wnt signaling in PPNAD that has been suggested by other studies (5, 15). The Wnt signaling pathway regulates a vast range of cellular functions such as growth and differentiation and is critical during embryonic development (16). In familial adenomatous polyposis, mutations of the adenomatosis polyposis coli (*APC*) gene activate the Wnt pathway and lead to the formation of

adrenal adenomas (17, 18). Interestingly, expression studies in both massive macronodular adrenocortical disease/adrenocorticotropic hormone–independent macronodular adrenal hyperplasia (19) and PPNAD (5) have indicated the overexpression of genes involved in the Wnt pathway such as *WISP2*, catenin-β1 (*CTNNB1*), and glycogen synthase kinase-3β (*GSK3B*).

Our findings here propose that microRNAs potentially link PKA and Wnt signaling pathways. The data suggest that microRNAs are a mechanism that might be involved in the formation of PPNAD nodules and the consequent activation of the Wnt pathway as shown in other studies (5, 15).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/15/09; revised 2/25/09; accepted 2/26/09; published OnlineFirst 4/7/09.

Grant support: NIH intramural project Z01-HD-000642-04 (C.A. Stratakis) and in part by the University of Crete, School of Medicine, Postgraduate Program (E.I. Bimpaki and C.A. Stratakis).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Carney JA, Gordon H, Carpenter PC, Shenoy BV, Go VL. The complex of myxomas, spotty pigmentation, and endocrine overactivity. Medicine 1985;64:270–83.
2. Stratakis CA. Cushing syndrome caused by adrenocortical tumors and hyperplasias corticotropin-independent Cushing syndrome. Endocr Dev 2008;13:117–32.
3. Kirschner LS, Carney JA, Pack SD, et al. Mutations of the gene encoding the protein kinase A type I-α regulatory subunit in patients with the Carney complex. Nat Genet 2000;26:89–92.
4. Boikos SA, Stratakis CA. Carney complex: pathology and molecular genetics. Neuroendocrinology 2006;83:189–99.
5. Horvath A, Mathyakina L, Vong Q, et al. Serial analysis of gene expression in adrenocortical hyperplasia caused by a germline *PRKAR1A* mutation. J Clin Endocrinol Metab 2006;91:584–96.
6. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–31.
7. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–8.
8. Thum T, Galuppo P, Wolf C, et al. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation 2007;116:258–67.
9. Nesterova M, Bossis I, Wen F, Horvath A, Matyakhina L, Stratakis CA. An immortalized human cell line bearing a *PRKAR1A*-inactivating mutation: effects of overexpression of the wild-type Allele and other protein kinase A subunits. J Clin Endocrinol Metab 2008;93:565–71.
10. Huang JC, Babak T, Corson TW, et al. Using expression profiling data to identify human microRNA targets. Nat Methods 2007;4:1045–9.
11. Johnson CD, Esquela-Kerscher A, Stefani G, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 2007;67:7713–22.
12. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 2008;10:593–601.
13. Kulshreshtha R, Ferracin M, Wojcik SE, et al. A microRNA signature of hypoxia. Mol Cell Biol 2007;27:1859–67.
14. Stratakis CA. Cushing syndrome caused by adrenocortical tumors and hyperplasias (corticotropin-independent Cushing syndrome). Endocr Dev 2008;13:117–32.
15. Stratakis CA. New genes and/or molecular pathways associated with adrenal hyperplasias and related adrenocortical tumors. Mol Cell Endocrinol 2009;300:152–7.
16. Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest 2002;109:987–91.
17. Naylor EW, Gardner EJ. Adrenal adenomas in a patient with Gardner's syndrome. Clin Genet 1981;20:67–73.
18. Bertherat J, Groussin L, Bertagna X. Mechanisms of disease: adrenocortical tumors-molecular advances and clinical perspectives. Nat Clin Pract Endocrinol Metab 2006;2:632–41.
19. Bourdeau I, Antonini SR, Lacroix A, et al. Gene array analysis of macronodular adrenal hyperplasia confirms clinical heterogeneity and identifies several candidate genes as molecular mediators. Oncogene 2004;23:1575–85.