UNIVERSITY OF CRETE
SCHOOL OF HEALTH SCIENCES

GRADUATE PROGRAM IN
THE MOLECULAR BASIS OF HUMAN DISEASE

Section on Endocrinology and Genetics (SEGEN), Program in Developmental Endocrinology & Genetics (PDEGEN), National Institute of Child Health & Human Development (NICHD)

Constantine A. Stratakis, MD, D (Med) Sci

Protein enzymatic changes of the cAMP pathway and microRNA aberrations in the pathogenesis of adrenocortical tumors

Master Thesis

Eirini I. Bimpaki, MD

Bethesda, MD 2008

ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ
ΚΥΤΤΑΡΙΚΗ ΚΑΙ ΓΕΝΕΤΙΚΗ ΑΙΤΙΟΛΟΓΙΑ
ΔΙΑΓΝΩΣΤΙΚΗ ΚΑΙ ΘΕΡΑΠΕΥΤΙΚΗ ΤΩΝ ΑΣΘΕΝΕΙΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ

Τομέας Ενδοκρινολογίας και Γενετικής (SEGEN),

Πρόγραμμα στην αναπτυξιακή Ενδοκρινολογία και Γενετική (PDEGEN),

Εθνικό Ινστιτούτο της Υγείας του Παδιού και της Ανάπτυξης του Ανθρώπου (NICHD)

Κωνσταντίνος Στρατάκης, MD, D (Med) Sci

Ενζυματικές αλλαγές στο cAMP μονοπάτι σηματοδότησης και αλλαγές στην έκφραση των microRNA στην παθογένεση των όγκων του φλοιού των επινεφριδίων

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Ειρήνη Ι. Μπιμπάκη, Ιατρός

Bethesda, MD, 2008

I would like to dedicate my master thesis

to my fiancé Babis Psarologakis

and to my parents Frosso and Yiannis Bimpakis

for their love, understanding and full support

during this challenging year.

Thank you for always being there for me.

Acknowledgements

Firstly, I would like to thank my supervisor, Doctor Constantine Stratakis, for all his help and support during my Master. Thank you for the inspiration, advice and the interesting conversations that we shared. Your doors have always been open to me, it is very much appreciated.

I would like to thank especially Dr Dimitrios Iliopoulos for all his expert advice and indispensable input on microRNAs experiments and for his support.

I would also like to thank our staff scientist, Dr Maria Nesterova, for her valuable help and advice during this project.

Also, I would like to thank Dr Zannis, Dr Boumpas, Dr Kardassis, Dr Goulielmos and Dr. Tsatsanis for their advice, help and support during my master.

Thanks to Dr. Sosipatros Boikos for all his support, assistance and advice throughout my master. A special thank you goes to Mrs Yianna Marinos, for her full support and advice during my Master and true friendship. Also, I would like to thank Dr Ariadne Androulidaki for helping me during my first steps in the bench work.

Big thanks go to all members, past and present, of the lab! Thanks to Dr. Anelia Horvath, to Kitman Tsang, to Dr. Audrey Robinson – White, to Dr. Limor Drori-Herishanu and to Jason Papademetriou. Also, many thanks to Dr. Maya Lodish, Dr. Somya Verma, Dr. Mike Muchow, Michelle Harran for your advice and your friendship. I would also like to thank Dr. Fabio Faucz, Dr. Maria-Eleni Nikita and Dr. Anna Koutoulaki for all their help and support during my thesis write-up.

I would like to thank Mrs Ioanna and Mr Ioannis Psarologakis for their full support and love.
Special thanks go to Maria Moutafi and Dimitra Terzi as well as to all my classmates in the Master Program 2006-2008 for their friendship and support.

Finally, I would like to acknowledge the Manasaki scholarship for the financial support and the National Institute of Health for funding this project.

Περίληψη

Οι αμφοτερόπλευρες επινεφριδιακές υπερπλασίες (BAHs) πιθανόν να προκαλούνται από γονίδια που κωδικοποιούν για μόρια τα οποία συμμετέχουν στο cAMP μονοπάτι σηματοδότησης. Λίγα είναι γνωστά για το cAMP μονοπάτι σηματοδότησης στους όγκους του φλοιού των επινεφριδίων οι οποίοι δεν φέρουν μεταλλάξεις σε γνωστά γονίδια. Η μαζική μακροοζώδης φλοιοεπινεφριδιακή νόσος (MMAD) και η πρωτοπαθής κεχρωσμένη φλοιοεπινεφριδιακή νόσος (PPNAD), δύο μορφές BAHs, πιθανον να σχετίζονται με αποκλίσεις στην έκφραση των microRNAs.

Μελετήσαμε το cAMP μονοπάτι σηματοδότησης με ενζυματικές και μοριακές μεθόδους σε όγκους του φλοιού των επινεφριδίων οι οποίοι δεν φέρουν μεταλλάξεις. Επιπλέον, μας ενδιέφερε να μελετήσουμε το microRNA προφιλ στις MMAD και PPNAD και να ανιχνεύσουμε τα πιθανά ζευγάρια microRNA - γονιδίων που εμπλέκονται στην επινεφριδιακή ογκογένεση.

Μελετήσαμε δείγματα ιστών από 27 ασθενείς με όγκους του φλοιού των επινεφριδίων (ηλικίας 5 - 60 ετών) και τα συγκρίναμε με φυσιολογικό ιστό του φλοιού των επινεφριδίων. Όλα τα δείγματα ελέγχθηκαν για μεταλλάξεις στα γονίδια : GNAS, PRKAR1A, PDE11A, PDE8B. Μελετήσαμε τα επίπεδα cAMP, και την συγγένεια πρόσδεσης στο cAMP καθώς και την ενεργότητα της πρωτεινικής κινάσης Α (PKA) και των φωσφοδιεστερασών (PDEs). Επιπρόσθετα, μελετήσαμε 10 ασθενείς με MMAD (ηλικίας 39-60 ετών) και 10 ασθενείς με PPNAD (ηλικίας 5-41 ετών) με στόχο να καθορίσουμε το microRNA προφιλ, 4 φυσιολογικοί επινεφριδιακοί ιστοι χρησιμοποιήθηκαν σαν κοντρολ. Όλα τα δείγματα αναλύθηκαν με microRNA microarrays και τα αποτελέσματα αξιολογήθηκαν με Real Time PCR. Τα αποτελέσματα συνεκτιμήθηκαν με δεδομένα έκφρασης που υπήρχαν διαθέσιμα πάνω στα ίδια δείγματα MMAD, PPNAD με στόχο να αναγνωρίσουμε τα πιθανά ζεύγη microRNA - γονιδίων τα οποία εμπλέκονται στον σχηματισμό της επινεφριδιακής υπερπλασίας. Χρησιμοποιώντας μια PPNAD κυτταρική σειρά, προσπαθήσαμε να διερευνήσουμε τους ρυθμιστικούς μηχανισμούς που ρυθμίζουν την έκφραση του miR-449.

Όλα τα αδενώματα ήταν αρνητικά για μεταλλάξεις, και είχαν χαμηλότερα επίπεδα PDE δραστηριότητας και υψηλότερη συγγένεια πρόσδεσης στο cAMP από το φυσιολογικό ιστό. BAHs με ή χωρίς μεταλλάξεις είχαν υψηλότερα επίπεδα cAMP και μειωμένη ελεύθερη - PKA ενεργότητα.

37 και 44 microRNAs είχαν διαφορετική έκφραση μεταξύ της MMAD και PPNAD αντίστοιχα και του φυσιολογικού επινεφριδιακού ιστού. Επίσης, αναγνωρίσαμε πιθανούς γονιδιακούς στόχους γι αυτά τα microRNAs. Μετά από συσχέτιση με διάφορες κλινικές παραμέτρους , θεωρήσαμε τα miR-130a και miR-382 καθώς και το let-7b ως πιθανούς διαγνωστικούς δείκτες στις MMAD και PPNAD αντίστοιχα. Επιπρόσθετα δείχνουμε ένα ρυθμιστικό μηχανισμό σύμφωνα με τον οποίο η PKA τροποποιεί την έκφραση του miR-449 καθώς και του γονιδίου στόχου WISP2.

Οι όγκοι του φλοιού των επινεφριδίων ανεξάρτητα με την ύπαρξη ή όχι μεταλλάξεων, επέδειξαν λειτουργικές ανωμαλίες στο cAMP μονοπάτι σηματοδότησης. Πιθανολογείται οτι επιγενετικά γεγονότα, επίδραση των microRNAs ή άλλα επιπρόσθετα γονιδιακά ελλείμματα είναι υπεύθυνα. Τα microRNAs φαίνεται να έχουν ιδιαίτερο ρυθμιστικό ρόλο στις MMAD και PPNAD, συμπεριλαμβανομένης μιας συσχέτισης με την κλινική εικόνα και την σοβαρότητα της νόσου. Επίσης, διαφαίνεται ότι τα microRNAs είναι ρυθμιστές του Wnt σηματοδοτικού μονοπατιού, εισάγοντας ένα νέο παθογενετικό μηχανισμό στην επινεφριδιακή ογκογένεση.

Abstract

Context Bilateral adrenal hyperplasias (BAHs) may be caused by mutations of genes that code for molecules that participate in cAMP signaling. Little is known about cAMP signaling in adrenocortical tumors (ADTs) that do not harbor mutations in known genes. Massive macronodular adrenocortical disease (MMAD) and Primary pigmented adrenocortical disease (PPNAD), two forms of BAHs may be caused by aberrant microRNA expression.

Objective We assessed the cAMP signaling pathway by enzymatic and molecular studies in mutation-negative ADTs. Furthermore, we were interested in identifying the microRNA profile in MMAD and PPNAD and in detecting putative microRNA-gene target pairs involved in adrenal tumorigenesis.

Design Samples from 27 patients with ADTs (ages 5-60 years) were studied and compared to normal adrenocortical tissue. All samples were sequenced for *GNAS*, *PRKAR1A*, *PDE11A*, and *PDE8B* sequencing defects. Cyclic AMP (cAMP) levels and binding, protein kinase A (PKA), and phosphodiesterase (PDE) activities were assayed. Additionally, 10 patients with MMAD (ages 39 - 60 years) and 10 patients with PPNAD (ages 5-41 years) were studied in order to identify the microRNA profile; 4 normal adrenal cortices were used as controls. All samples were analyzed using microRNA microarrays; data were validated by Real Time PCR. MicroRNA microarray analysis was integrated with expression data in the same MMAD, PPNAD samples to identify potential microRNA-gene target pairs implicated in adrenal hyperplasia formation. Using a PPNAD cell line we tried to investigate the regulatory mechanisms underlying the expression of miR-449.

Results All adenomas were mutation-negative; these tumors had lower PDE activity levels and higher cAMP binding affinity than normal adrenal. BAHs, both with and without any mutations, had higher cAMP levels and decreased free PKA activity.

A total of 37 microRNAs, 44 microRNAs were differentially expressed between MMAD, PPNAD and normal tissues respectively. We also identified putative gene targets for these microRNAs. After considering various clinical parameters from our patients, we identified miR-130a and miR-382 as putative diagnostic markers in MMAD and let-7b in PPNAD. We demonstrate a regulatory mechanism according to which PKA modulates the expression of miR-449 and its target gene WISP2.

Conclusion: ADTs independently of their mutation status, demonstrated functional abnormalities of cAMP signaling. It is probable that epigenetic events, microRNA

implication or additional defects of genes involved in this pathway are responsible. MicroRNAs appear to have distinct regulatory effects in MMAD and PPNAD, including an association with clinical presentation and severity of the disease. MicroRNAs are modulators of the Wnt signaling pathway, indicating a novel pathogenetic mechanism in adrenal tumorigenesis.

Table of Contents

List of figures

List of Tables

List of Abbreviations

17-OHS	17-Hydroxy-steroid
ACA	Adrenocortical Adenoma
ACC	Adrenocortical Cancer
ACS	atypical Cushings Syndrome
ACTH	adrenocorticotropic hormone
AD	autosomal dominant
AIMAH	adrenocorticotropin-independent macronodular adrenocortical hyperplasia
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
BADX	bilateral adrenalectomy
BMAH	bilateral macroadenomatous hyperplasia
BWS	Beckwith–Widemann syndrome
cAMP	cyclic adenosine monophosphate
CHEK2	checkpoint 2 gene
CNC	Carney complex
CPA	cortisol producing adenoma
c-PPNAD	CNC-associated PPNAD
CREB	cAMP response element binding
CRH	corticotropin releasing hormone
CS	Cushing Syndrome
FAP	familial adenomatous polyposis
FH	fumarate hydratase
GDP	guanosine diphosphate;
GNAS	gene coding for the stimulatory subunit α of the G-protein (Gsα)
GPCR	G-protein-coupled receptor
GSK-3	glycogen synthase kinase 3
Gsα	G-protein α subunit
GTP	guanosine triphosphate
Gβ	G-protein β subunit
Gγ	G protein γ subunit

HLRCS	hereditary leiomyomatosis and renal cancer syndrome
IGF2	insulin-like growth factor II gene
i-MAD	isolated micronodular adrenocortical disease
INHA	inhibin A gene
i-PPNAD	isolated PPNAD
LFS	Li–Fraumeni syndrome
LRP	lipoprotein receptor–related protein
MAS	McCune–Albright syndrome
MEN1	multiple endocrine neoplasia type 1
NR5A1	nuclear receptor subfamily 5 group A member 1
ORF	open reading frame
P	Phosphate
PDE11A	phosphodiesterase 11A gene
PKA	protein kinase A
PPNAD	primary pigmented nodular adrenocortical disease
PRKACa	A catalytic subunit of PKA
PRKAR1A	protein kinase regulatory subunit 1 of PKA
RISC	RNA-induced silencing complex
RTS	Rubinstein–Taybi syndrome
TCF/LEF	T cell–specific transcription factor/lymphoid enhancer-binding factor
TP53	tumor protein p53 gene
UADX	unilateral adrenalectomy
UFC	Urinary Free Cortisol
WISP2	WNT1-inducible signaling pathway protein 1
WNT	wingless-type MMTV integration site family

1 Introduction

1.1 Adrenal Glands; Anatomy and function

Adrenal glands are two triangular endocrine glands, responsible for many critical functions of the human body. They are situated anteriosuperiorly to the kidneys (figure1.1), on the level of the 12th thoracic vertebra**;** their blood supply is provided by the adrenal arteries. Histopathologically, they can be divided into the adrenal medulla, the central part, and the adrenal cortex. The adrenal medulla is the main source of cathecholamines: adrenaline and nor-adrenaline that mediate the fight or flight response via the sympathetic nervous system. The adrenal cortex is mainly responsible for 3 major functions: glucocorticoid synthesis (cortisol), mineralocorticoid synthesis (aldosterone) and androgen synthesis (testosterone, androstenedione). Cortisol production is regulated via the hypothalamic-pituitary-adrenal axis under the control of ACTH (Adrenocoticotropic Hormone). (1,2)

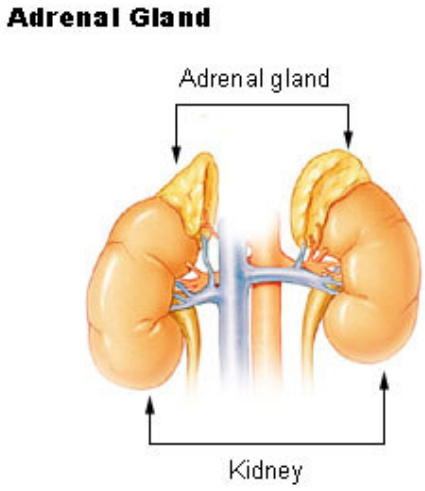


Figure 1.1 Adrenal glands (Grays Anatomy)

Adrenal dysfunction may lead to obesity, metabolic syndrome, osteoporosis and late onset diabetes- mellitus. Nowadays, the increasing use of Computed Tomography for diagnostic purposes has led to the estimation that almost one in ten adults has a nodule (> 1 cm) in the adrenal cortex (3). These incidentalomas are most commonly classified as adrenal adenomas; benign unilateral adrenal tumors that bear a good outcome. Adrenal cancer is rare with an incidence of 1 in 1500 of the adrenocortical

tumors and a prevalence of 4-12 cases per million (4) but is associated with a high morbidity rate in case it remains untreated. Bilateral adrenal hyperplasias (BAHs) comprise a rather less well known group of adrenal disorders with an important role in the pathogenesis of Cushing Syndrome. Mainly, Primary Pigmented Nodular Adrenocortical Disease (PPNAD) and ACTH Independent Macronodular Adrenocortical Hyperplasia (AIMAH), both familial and sporadic forms, have been implicated in Cushing Syndrome (3).

1.2 Cushing Syndrome

Cushing syndrome (CS) is an endocrine syndrome caused by high levels of cortisol in the serum. It may be due to pituitary adenomas; a medical condition known as Cushing's disease, due to ectopic excretion of adrenocorticotropic hormone (ACTH) or due to ACTH-independent adrenal tumors. In children, it usually presents with weight gain and delayed growth development. Other symptoms include facial plethora, hypertension, hirsutism and skin manifestations such as striae and acanthosis nigricans. In order to establish the diagnosis of Cushing, hypercortisolism should be documented. Usually, the 24h urinary free cortisol (UFC) excretion is measured (normal range: $<70\mu g/m^2/24h$). A gold standard test that is being used very often in order to confirm endogenous excess of cortisol is the diurnal plasma cortisol variation. Midnight cortisol levels higher than 5μg/dl are indicative of CS. Other tests such as corticotropin releasing hormone (CRH) test and the Liddle's test are performed in order to distinguish the cause of Cushing's syndrome (1,5).

1.3 Adrenal Cushing Syndrome

Bilateral adrenocortical hyperplasias, along with the most common adrenal adenoma and the very rare adrenal carcinoma comprise the spectrum of diseases that can be the causes of adrenal Cushing's syndrome.

1.3.1 Bilateral Adrenocortical Hyperplasias (BAHs)

It is estimated that almost 10% in young adulthood and 15% in childhood, of the ACTH-independent Cushing's syndrome can be due to BAHs. The most known groups of adrenal hyperplasia are the primary pigmented nodular adrenocortical disease (PPNAD) and the ACTH - independent macronodular adrenocortical hyperplasia (AIMAH) which is also known as massive macronodular adrenocortical

disease (MMAD). Recent findings, mainly in the field of molecular genetics, have added more knowledge regarding the existence of different forms of adrenal hyperplasias and a new nomenclature has been recently suggested by Stratakis et al, 2007, (6). Three main criteria have been used for this classification. The size of the nodules (1cm is the size-limit that distinguishes micro- from macronodular hyperplasias), the presence of hyperplasia or atrophy in the surrounding tissue as well as the detection of pigmented lesions (Table1.1) (6).

Adrenocortical lesions	Age-group affected	Histopathology	Associated disease and inheritance	Protein, gene or locus affected
Benign				
Common adenoma	All ages	Adenoma of the zona fasciculata	MEN1, FAP, MAS, HLRCS, CNC, Carney triad and others	Menin, *APC*, *GNAS*, FH, *PRKAR1A*, 2p16, 9q34, *INHA*, *TP53*, GPCRs and others
Macronodular hyperplasias (multiple nodules, most >1 cm diameter)				
BMAH	Middle age	Distinct adenomas (usually two or three), with internodular atrophy	MEN1, FAP, MAS, HLRCS, other, isolated (AD) and others	Menin, FH, ectopic GPCRs, *APC* and *GNAS*
c-BMAH	Infants and very young children	As for BMAH; occasional microadenomas	MAS	*GNAS*
AIMAH	Middle age	Adenomatous hyperplasia (multiple), with internodular hyperplasia of the zona fasciculata	Isolated (AD)	Ectopic GPCRs; WISP2 and WNT signaling, 17q22-24, *GNAS* and others
Micronodular hyperplasias (multiple nodules, most <1 cm diameter)				
i-PPNAD	Children and young adults	Microadenomatous hyperplasia, with (mostly) internodular atrophy and nodular pigment (lipofuscin)	Isolated (AD)	*PRKAR1A*, *PDE11A*, 2p16 and others
c-PPNAD	Children; young and middle-aged adults	Microadenomatous hyperplasia, with (mostly) internodular atrophy and (mainly nodular) pigment (lipofuscin)	CNC (AD)	*PRKAR1A*, 2p16 and others
i-MAD	Mostly children and young adults	Microadenomatous, with hyperplasia of the surrounding zona fasciculata and limited or absent pigment	Isolated (AD); occasionally part of other developmental defects	*PDE11A*, 2p12-p16, 5q and others
Malignant				
Sporadic cancer	All ages	Mitotic figures, atypia of cortical cells, capsular invasion and metastases	Isolated	β-Catenin, *TP53*, *INHA*, 2p, 2q, 9q, 11q and others
Syndromic cancer	Children and young adults	As for sporadic cancer	LFS (AD), BWS, RTS and others	*TP53*, *CHEK2*, *IGF2* and others
Brazil variant	Children and young adults	As for sporadic cancer, but a milder clinical course	AD and others	*TP53*, *INHA*, NR5A1, 9q34 amplification and others

Table 1.1: Adrenocortical lesions causing Cushing's syndrome (6)

1.3.1.1 PPNAD

Primary pigmented nodular adrenocortical disease (PPNAD) is a rare form of bilateral adrenal hyperplasia that can cause CS. PPNAD is linked to Carney Complex (CNC); an autosomal dominant disease with multiple neoplasia manifestations from the endocrine system that was first described in 1985 by Dr J. Aidan Carney (7) (see below CNC). PPNAD also exists in its sporadic form, but less frequently; almost 90% of PPNAD cases are associated with CNC. This form of adrenal hyperplasia is the

most frequent endocrine tumor in patients with CNC and it can present either in the first years of life or more commonly in the 2-3 decade of life. It can be differentially diagnosed form other causes of hypercorisolism using Liddle's test; a paradoxical increase in urinary free cortisol and 17-OH-Steroid is detected on day 6 after dexamethasone stimulation (5).

PPNAD is attributed to inactivating mutations of the regulatory subunit R1A of the PKA enzyme that lead to non functional protein products. According to biochemical studies on PKA function, PPNAD tumors exhibit increased PKA activity upon cAMP stimulation (8) mainly attributed to overexpression of other regulatory subunits of the PKA protein (9).

Recently, the gene expression profile was studied in PPNAD, using serial analysis of gene expression (SAGE) and revealed the differential expression of several important genes implicated in steroidogenesis and cell proliferation signaling pathways, between PPNAD and normal adrenal (10).

Pathologic features of the adrenals in PPNAD include small or normal size of the gland and the presence of black or dark brown nodules throughout the atrophic cortex. Especially in young patients the combination of the atrophic cortex and the pigmented micronodules is diagnostic. In older patients, nodules more than 1cm have been detected (3).

1.3.1.2 iMAD

iMAD is a micronodular adrenal hyperplasia and although it has many common features with PPNAD, it consists a distinct entity. It usually has an early onset and presents with atypical Cushings Syndrome (ACS). Patients with ACS have a hectic phenotype accompanied be osteoporosis and muscle wasting. The urinary free cortisol levels in these patients tend to range between normal values (11). This type of adrenal hyperplasia has been associated with mutations in the PDE11A gene, according to a genome wide screen of 10 kindreds (12). The pathologic features of the disease include hyperplasia of the surrounding adrenal cortex and absence of pigmentation (6).

1.3.1.3 MMAD/AIMAH

Unlike the case with PPNAD, most patients with MMAD do not have family history of this disease and present with CS in middle age or even late adulthood (13). Aberrant expression of G protein-coupled receptors (GPCRs) was identified as the main molecular mechanism that underlies the cause of MMAD in the majority of the cases (14), but how this molecular abnormality is generated in the adrenal cortex of affected subjects remains the subject of intense inquiry.

Somatic mutations of the *GNAS* gene are present in patients with McCune-Albright syndrome (MAS) and CS due to BAH (15); in addition, a number of patients with MMAD/AIMAH were found to harbor *GNAS* mutations in their adrenocortical tissue (16). In these cases, aberrant GPCR expression may be due to activation of the protein kinase A (PKA) signaling pathway by increased cAMP levels (3) but in most patients with MMAD/AIMAH the underlying cause of this phenomenon remains a mystery. Our laboratory reported several genes that may play a role, following an expression microarray analysis of tissues affected by MMAD/AIMAH (17).

1.3.2 Adrenocortical Adenoma

Adrenortical adenoma (ACA) is the most common cause of adrenal Cushing's syndrome. Usually it is unilateral, it can present at all ages and it has been associated with the Multiple Endocrine Neoplasia syndrome type 1 (MEN1) (approximately 50% of the patients diagnosed with MEN1 have ACAs), with Familial Adenomatous Poluposis syndrome (FAP), McCune-Albright and others. Despite its benign nature, ACA also has to be removed laparoscopically in symptomatic patients (6).

1.3.3 Adrenocortical Cancer

Adrenocortical cancer (ACC) is quite rare, the estimated prevalence rate is 4-12/ million of population and bears a poor prognosis (18). In order to differentiate the ACC from the adrenocortical adenoma we currently use the Weiss score which is based on histopathological features of the tumor such as necrosis, architecture and vascular invasion (19). The required treatment for ACC is open adrenalectomy, although most of the patients are diagnosed in a late stage when metastases are already present (20). ACC has been linked with several familial disorders such as Li-Fraumeni syndrome and Beckwith-Wiedemann syndrome (21).

1.4 The cAMP signaling pathway

1.4.1 GSA and MAS

During the investigation of adrenal tumorigenesis it has been indicated that aberrations of the cAMP pathway have a major role (figure 1.2). The first element of this pathway that was associated with the pathogenesis of adrenocortical tumors was the stimulatory Gs-protein a subunit (encoded by GNAS1), which was found altered in McCune-Albright syndrome.

G proteins are cardinal mediators of extracellular signals into cellular responses. They are heterotrimeric compounds of three subunits: alpha, beta and gamma, which are encoded by multiple different genes. In the inactive state, a subunit is bound to GDP and to the heterodimer of beta and gamma subunits. Upon activation, GDP is replaced by GTP and a subunit dissociates from the beta, gamma dimmer and the recetor. At this phase, a subunit activates ion channels and other effector molecules until it is inactivated again by its endogenous GTPase activity, returning to the non-stimulated stage (21-23).

G proteins, due to their variability of their subunits, can interact with a great range of receptors. Adenylate Cyclase is activated by receptors bound to G alpha s (alpha subunits with a ubiquitous expression) leading to increased cAMP synthesis and PKA activation. Gsa subunit is encoded by the GNAS1 gene located in chromosome 20q13.1-13.2. Mutations of this gene may lead to the stabilization of G protein in either the active or the inactive state (21).

McCune-Albright Syndrome is a sporadic disease, which was first described in 1936 by McCune and Albright with a series of case reports. It is caused by postzygotic activating mutations of the a-subunit of G protein that lead to an increase in Adenylate Cyclase activity in a monoclonal cellular population. The wide distribution of the cells which bear the activating mutation defines the features of the disease, depending on the different tissues which are involved. In the majority of the cases, the mutation is a substitution of Arginine 201 by histidine or cysteine, that leads to the stabilisation of Gsa protein in an active form. The main three characteristics of this syndrome include polyostotic fibrous dysplasia, café au lait spots and precocious puberty,

nevertheless other characteristics have been also reported. Endocrine disorders such as secreting pituitary adenomas, adrenal hyperplasia and hypercortisolism, thyroid and parathyroid dysfunction and osteomalacia have been associated with MAS, suggesting that it is a quite heterogeneous condition with a wide range of manifestations. Hypercortisolism due to adrenocortical tumors is a rare finding in patients with MAS, and it has mainly been described during the early years of life. According to a study of 113 patients with signs of MAS, 7 of them had high levels of cortisol, and the activating mutation in Gsa was detected in two out of the four adrenal tissue samples available for testing (22). Cushing's in MAS can resolve spontaneously, but most of the times treatment is required; adrenalectomy is the treatment of choice . The correlation of Gsa mutations and the adrenal dysfunction in patients with MAS has established a possible pathogenetic mechanism for adrenal tumorigenesis. Is that mechanism also present in sporadic forms of adrenal disease? In patients with Cushing's syndrome, due to AIMAH, and without any typical signs of MAS, activating mutations of Gsa have been detected (23).

1.4.2 PRKAR1a and CNC

Protein kinase A is another core member of the cAMP signaling pathway that is involved in adrenal tumorigenesis. Inactivating mutations of the regulatory subunit 1 of PKA protein has been described in patients with Carney Complex, a multiple endocrine neoplasia syndrome.

Protein kinase A is cAMP dependent kinase, with a regulatory role in cell proliferation, growth, transcription and apoptosis. PKA consists of two homo- or hetero-dimmers of regulatory subunits and two catalytic subunits. The regulatory subunits exist in two forms, RI and RII and combine with the catalytic subunit (Ca, Cβ or Cγ) in order to form the PKA molecule. PKA can be detected in human cells in two at least different isoforms depending on the identity of the homo/heterodimers of the regulatory subunits. PKA I consists of the RIa and/or RIb and PKA II respectively consists of RIIa and/or RIIb subunits. RIa is the most abundant subunit and is encoded by the PRKAR1A gene, located in 17q22-24 locus (24).

The inactive form of PKA consists of the two regulatory subunits and the two catalytic ones. Regulatory subunits comprise of the amino terminal dimerization

domain, a region that interacts with the catalytic subunit, and the carboxyl terminus domain. Two cAMP binding sites, site A and site B are located in the carboxyl terminus domain. Binding of cAMP to site B, changes the conformation of the molecule and enhances the binding of cAMP to site A (24). So, upon activation of the molecule, two cAMP molecules bind to each R subunit, resulting in the dissociation of the catalytic subunits. The catalytic subunits have serine-threonine kinase activity and are capable of phosphorylating a wide range of transcriptional factors and other substrates, regulating cell growth and differentiation. Inactivating mutations of the R1A subunit of PKA have been identified in the majority of patients with Carney Complex (9).

Carney Complex (CNC; MIM# 160980) is a dominantly inherited disease with three main characteristics: spotty skin pigmentation (leniginosis), myxomas (cardiac and cutaneous) and endocrine hyperactivity. Most of the patients previously diagnosed under the syndromes of LAMB (lentigines, atrial myxoma, blue nevi) or NAME (nevi, atrial myxoma, myxoid neurofibroma, ephelide) are now characterized as CNC patients.
Although some of the features may present early in life (lentigines, myxomas) most of the patients are diagnosed in the second or third decade of life. Cardiac myxomas can appear at any age, are more aggressive in comparison to sporadic myxomas and are responsible for 50% of the mortality in CNC patients. Cutaneous myxomas include myxomas of the median ear, eyelid, breast as well as female genital system.

Various endocrine disorders have been described in CNC patients. The most common feature is primary pigmented nodular adrenocortical disease (PPNAD), a form of adrenal hyperplasia causing CS. ACTH-independent Cushing's syndrome is the main endocrine manifestation in CNC patients. Approximately one fourth of CNC patients, have been diagnosed with PPNAD and this might be an underestimation due to subclinical and periodical forms of CS. Other endocrine disorders include thyroid gland dysfunction, pituitaty adenomas as well as testicular tumors. Life expectancy is decreased in these patients, mainly because of the cardiac myxomas and neoplasias (25).

Genetic linkage studies have identified two different loci associated with this syndrome, 17q22-24 (CNC1) and 2p16 (CNC2). Locus 2p16 is still under investigation, since no specific gene has been indicated yet. Hypothetically, a non coding molecule could be related with this locus eg a miRNA. According to a study in CNC patients registered in the NIH Mayo Clinic collection, 52.8% of the kindreds had a mutation in CNC1 locus (9). Until today, approximately 60 PRKAR1A inactivating mutations have been detected; most of them are substitutions, small deletions or insertions. Only two large deletions have been described in two patients with CNC (26). In the majority of the cases the mutations lead to a premature stop codon and mRNAs are degraded by nonsense mediated mRNA decay. Additionally, loss of hybridization for PRKAR1A was identified in tumors from CNC patients, suggesting the putative role of R1A as a tumor suppressor gene (8).

Recently, mutations that escape nonsense mediated mRNA decay and lead to the expression of truncated, malfunctioning proteins have been described (26, 27). In vitro studies regarding those mutations have reported increased PKA activity, decreased cAMP binding and increased pCREB / CREB ratios indicating that impaired PRKAR1A function is sufficient for alterations in PKA activity that enhance tumorigenesis (27).

PRKAR1A mutations have also been detected in patients with isolated PPNAD, with no other symptoms of Carney Complex; most probably they have occurred de novo (4, 9).

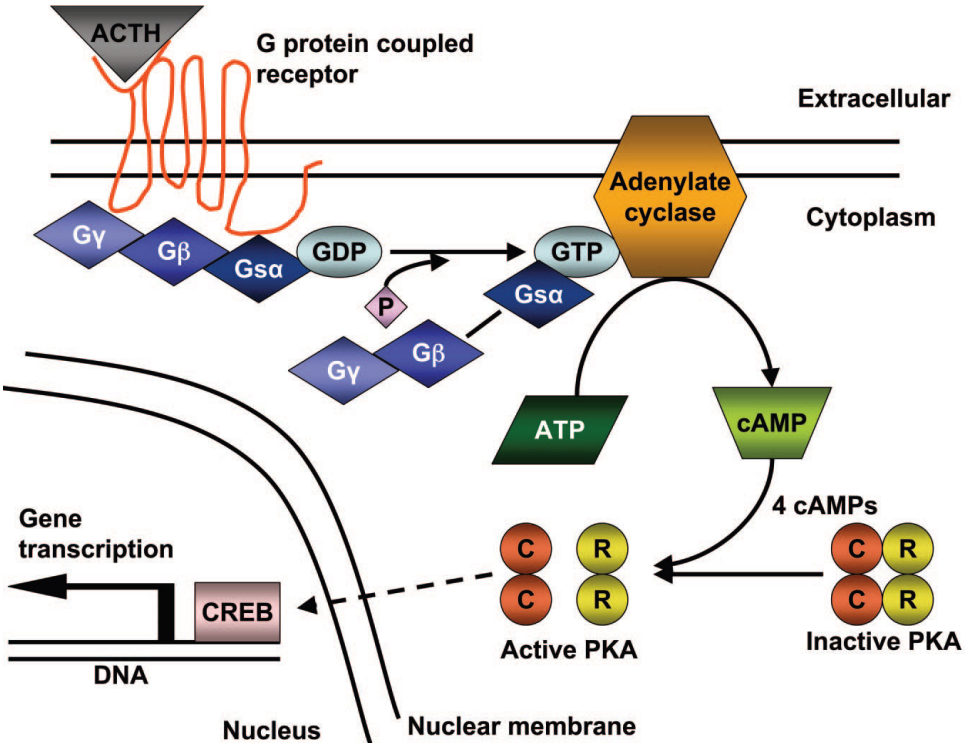


Figure 1.2: The ACTH–cAMP–PKA pathway (18).

1.4.3 PDE11A

Additionally to GSA and PRKAR1A, another molecule associated with the cAMP pathway has been found implicated in adrenal tumorigenesis. Inactivating mutations of the phosphodiesterase 11A (PDE11A) have been detected in a genome wide screen of 10 kindreds, with adrenal hyperplasia and without any other known defects, like PRKAR1A or GNAS mutations (12).

Cyclic nucleotide phosphodiesterases (PDEs) are the enzymes, which degrade the cAMP and/or cGMP molecules and inactivate them, regulating cell signaling and cell homeostasis. PDEs comprise a huge group of enzymes; over 100 different proteins have been reported until today. The vast variety of these enzymes is attributed not only to the large number of genes that encode these proteins (21 genes) but also to their complex structure. Most of these genes are regulated by multiple promoters and in combination with alternative splicing, a big variety of transcripts is expressed (28).

PDEs can be divided in 11 families (PDE1-PDE11) based on their sequence homology and their biochemical and pharmacological and features. Additionally, according to their substrate specificity they can be subdivided into those that are cAMP specific (PDEs 4,7,8), cGMP specific (PDEs 5,6,9) and those that can hydrolyze both cAMP and cGMP (PDEs 1,2,3,10,11) (28). According to structural studies on PDEs, the specificity of their catalytic site is mainly regulated by the orientation of a glutamine residue located in the binding pocket of the enzyme. When the rotation of this residue is not constrained by adjacent molecular bonds, then the enzyme can hydrolyze both cyclic nucleotide phosphates whereas, if the orientation of glutamine is stable, the enzyme has cAMP or cGMP specifity depending on the conformation established (29).

PDE11 family has been reported for the first time by Fawcett et al, 2000 and is the most recently described one (30). The family consists of one gene product, PDE11A, with 4 different variants (A1-4). PDE11A gene is located in chromosome 2, locus 2q31.2 and contains 23 exons. The 4 variants are differentially expressed among tissues, and the findings regarding their expression pattern are contradictory. According to a recent study all PDE11A variants were detected in the prostate, testis, kidney, adrenal, colon and epidermis (31) although, other studies showed no expression of the isoforms in the human testis (32). PDE11A1 is the most ubiquitously expressed and PDE11A2, A3 isoforms are mainly expressed in the testis. PDE11A4 isoform is most highly expressed in the prostate and has also been found expressed in the pituitary, heart, liver and the adrenal gland (33,34).

Several PDE aberrations have been associated with human inherited diseases, such as PDE4D polymorphisms and increased stroke susceptibility, PDE4B alleles and schizophrenia and PDE6 mutations and congenital eye disease. No endocrine disorder has been associated with malfunction of PDEs until the implication of PDE11A inactivating mutations in adrenal hyperplasia (28,34).

The adrenal glands of patients with CS who bear the PDE11A mutations and PPNAD patients had similar features such as normal size and weight, multiple yellow to dark brown nodules and granules of lipofuscin. Despite the similar appearance with PPNAD, no PRKAR1A mutations were detected (12).

Five PDE11A mutations were reported: two frameshifts (171delTfs41X and 1655_1657delTCT/insCCfs15X) and one base pair substitution (R307X) that leads to a premature stop codon, which were protein-truncating and two missense substitutions (R804H and R867G). FISH studies indicated loss of heterozygosity with retention of the mutant allele in some of these patients. Furthermore, the cAMP and cGMP levels were analysed in tissue lysates from the adrenals, considering the putative effects of PDE11A inactivation in the degradation of the second-messenger molecules. Indeed, the cAMP and cGMP were elevated in the affected individuals in comparison with the normal adrenals. Also, the levels of phosphorylated CREB were increased in the adrenals that bear the PDE11A mutations. Those data indicated a putative pathophysiological mechanism in adrenal tumorigenesis according to which PDE11A inactivating mutations lead to aberrations in cAMP signaling. PDE11 is partially inhibited by Tadalafil (IC50: 73nM), a PDE5 inhibitor administered to patients with erectile dysfunction, but no side effects regarding the adrenal function have been reported so far (12).

The presence of the five germline PDE11A inactivating mutations in the general population was studied. The three truncating variants were also present in normal subjects, but with much lower frequency that in patients with adrenal hyperplasia. The other two missense mutations were detected in normal subjects with lower frequency than among patients with adrenal lesions but without statistical significance. The presence of the inactivating PDE11A mutations in the general population indicates their possible role as predisposing factors to adrenocortical neoplasms and other tumors (34).

Also the frequency of PDE11A alterations was studied in patients with other adrenocortical lesions, apart from micronodular adrenocortical disease such as AIMAH (or MMAD), ACA and ACC. PDE11A was screened in 117 adrenocortcal tumors and 192 controls; one inactivating mutation that was previously described (12) was found in ACA but not in the controls, 22 missense variants were detected in the tumor samples and 11 in the control group. The common coding sequence variants had a higher frequency in the adrenal lesions than in the controls. Among

adrenocortical tumors the higher frequency was detected in AIMAH (24%) in comparison with ACA (19%) and ACC (16%). This preference was anticipated since a genetic predisposing factor would be more frequent in a bilateral disease as AIMAH, in contrast to unilateral ACA and ACC. cAMP levels were also studied and in most cases of AIMAH and ACA, cAMP levels were higher than in normal adrenal, suggesting the malfunction of PDE11A in cAMP degradation. Immunocytochemistry in the tumor samples with the PDE11A missense variants showed decreased levels of PDE11A, indicated a role of this gene as a tumor suppressor in the adrenal cortex as previously suggested by Horvath et al, 2006.These findings present the implication of less deleterious mutations of PDE11A in various adrenocortical tumors, implying its putative role in the frequently discovered in the general population adrenal incidentaloma (35).

A recent study investigated the possibility that PDE11A and PRKAR1A interact by regulating each other through the cAMP pathway. In adrenal tissues with PDE11A mutations the PRKAR1A expression was higher than in normal adrenal. Also, in adrenal samples with PRKAR1A a defects, PDE11A levels were higher than in normal adrenals, although without statistical significance. Furthermore, different adrenal hyperplasias were compared regarding their CREB/pCREB levels. Patients with iMAD (PDE11A defects) had the highest pCREB/ CREB ratio. Based on these findings the underlying mechanism could be that in PDE11A defective tissues, cAMP levels would be expected to be higher, leading to increased activation of PKA, increased pCREB/CREB levels and even increased PRKAR1A levels due to the CRE sequences in the promoter of the gene. Increased PRKAR1A levels would than lead to inactivation of PKA and thus would inhibit the responce to increased cAMP levels. On the other hand, in tissues with PRKAR1A defects, where the PKA system would be stimulated and the cAMP signaling increased, elevation of the PDE levels such as PDE11A would degrade cAMP and would control the hyperactivation of the cAMP pathway(36).

1.4.4 PDE8B

The same genome wide study that identified the mutations in PDE11A, suggested as a candidate gene PDE8B due to its location in 5q13. A mutation ; c.914>T, pH305P has been detected in a young girl with Cushing syndrome and was associated with

abnormal PKA activity. This single base substitution was not found in the control group that was tested, giving another indication of the potential role of PDEs in adrenocortical function (11,37).

1.5 The WNT signaling pathway

Besides the cAMP pathway, another transduction system has been associated with adrenal pathogenesis; the Wnt signaling pathway. The Wnt signaling pathway regulates a vast range of cellular functions such as growth and differentiation and is critical during embryonic development (38). The major event in the Wnt signaling pathway is the phosphorylation of β-catenin (CTNNB1) by the glycogen synthase kinase -3β (GSK3B) that leads to the degradation of CTNNB1 and the downregualtion of the target genes. On the other hand, activation of the Wnt receptor leads to the inhibition of the GSK3B, the accumulation of CTNNB1 in the cytoplasm and its subsequent translocation into the nucleus that highlights the upregulation of the target genes (figure 1.3) (Soon, The Oncologist, 2008).

The first association between Wnt pathway and adrenal lesions was made in patients with Familial Adenomatous Polyposis (FAP) who also developed adrenal adenomas. In FAP, mutations of the adenomatosis polyposis coli (*APC*) gene activate the Wnt pathway (39). Interestingly, expression studies in both MMAD/AIMAH (17) and PPNAD (12) have indicated overexpression of genes involved in the Wnt pathway such as axin1 (*AXIN1*), WNT1-inducible signaling pathway protein 2 (*WISP2*), catenin- β1 (*CTNNB1*) and glycogen synthase kinase -3β (*GSK3B*). According to recent studies somatic mutations of β-catenin were found in both benign and malignant adrenocortical tumors leading to activation of the Wnt pathway (40, 41).

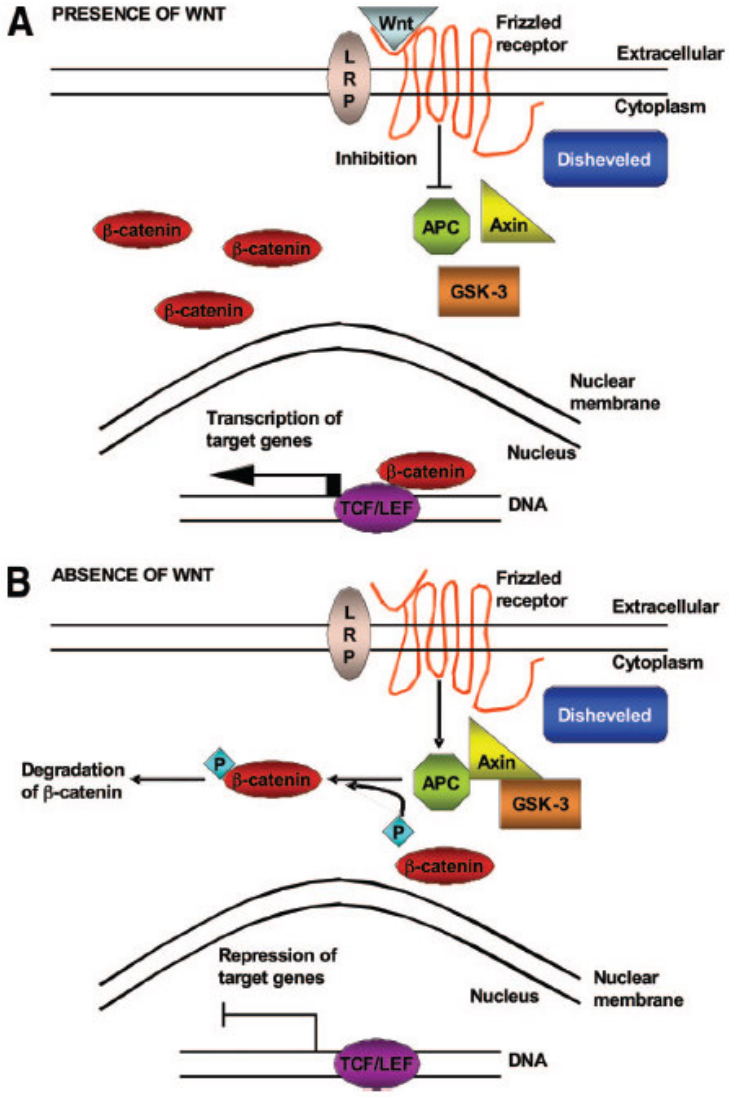


Figure 1.3: Regulation of Wnt signaling pathway.

(A): Presence of Wnt molecule. **(B):** Absence of Wnt molecule (18).

1.6 MicroRNAs

MicroRNAs are noncoding, single stranded, ~22 nucleotides in length, RNA sequences that have recently been identified as important regulators of target gene expression at the mRNA or protein level: if there is partial complementarity between the sequence of a given microRNA and that of its target gene, then the microRNA binds in the 3'UTR of the gene and regulates translation; if, on the other hand, the sequences of a microRNA and the target gene are perfectly complementary, then regulation takes place at the target gene's mRNA level resulting usually in cleavage and inhibition of trasncription (42-44). The precursor molecules of microRNAs (pri-miRNAs) are transcribed by nuclear polymerase II and are then processed into pre-miRNAs, by the enzymatic complex Drosha-DGCR8. Pre-miRNAs are 60 to 70 nucleotide sequences that form a hairpine structure and are transported from the nucleus into the cytoplasm through exportin 5 (43). Mature miRNAs are then generated after the cleavage of pre-miRNAs by Dicer, another RNase III (44). MicroRNAs exhibit their effects into the RISC complexes (RNA-induced silencing complex) where the mRNA target is suppressed (figure 1.4) (43). MicroRNAs exhibit a critical role in multiple cellular functions, such as growth and proliferation, cell cycle control, and apoptosis (44). Consequently, microRNAs have been implicated in the pathogenesis or progression of several diseases, including cancer (45-50), where they can act as tumor suppressors or oncogenes. Although widespread aberrations are indicated in different cancer types, it is well documented that several microRNAs play very specific roles in tumorigenesis regulating certain pathways (44-50).

Recently, microRNA array profiling has been used in the investigation of numerous clinical samples in an effort to identify those that are differentially expressed and study further their role in disease pathogenesis. Several types of proliferative diseases have been investigated, including chronic lymphocytic leukemia (45), pancreatic (46), breast (47), prostatic (48) and lung (49) cancer.

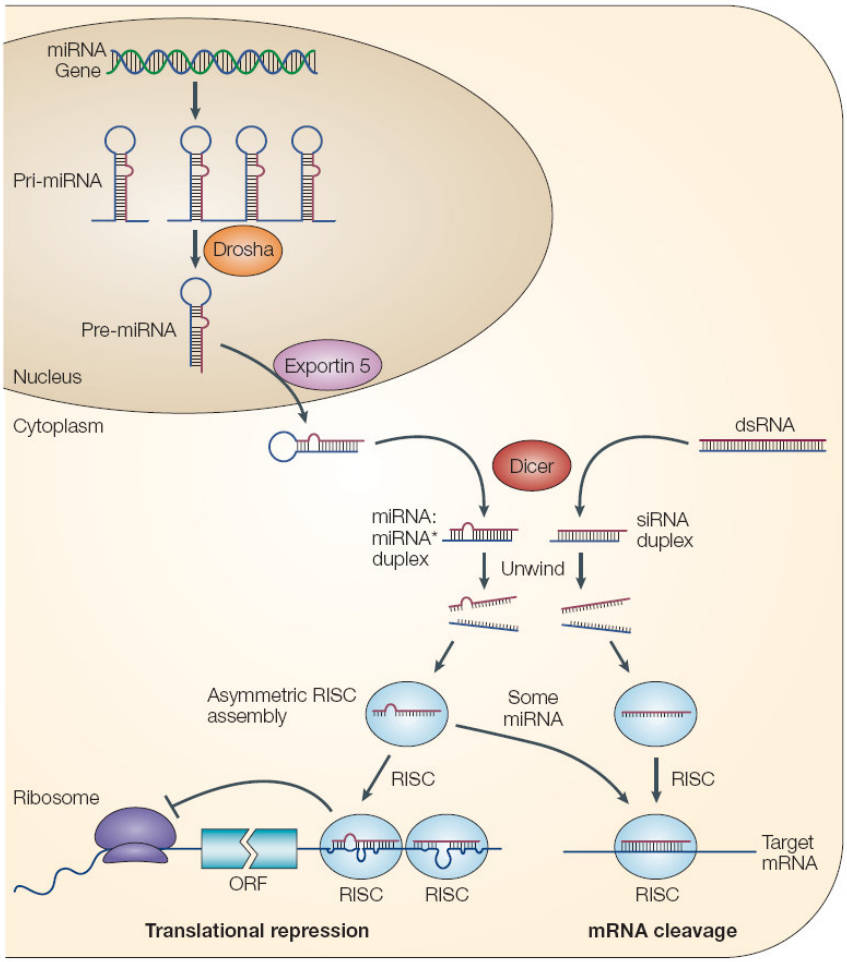


Figure 1.4: The biogenesis of microRNAs (50)

1.7 Overall aims of the thesis

The cAMP signaling pathway is involved in the pathogenesis of BAHs (6, 51): *GNAS* activating mutations were found in macronodular BAH in the context of McCune-Albright Syndrome (MAS) (52) and in massive macronodular adrenocortical disease (MMAD), (16) and G-protein coupled receptor aberrant expression has been found in both MMAD/AIMAH and sporadic ADTs, including CPAs (51, 53, 54). Mutations in the *PRKAR1A* gene coding for the regulatory (R) subunit 1A of cAMP-dependent protein kinase (PKA) are responsible for the most common form of micronodular BAH, primary pigmented nodular adrenocortical disease (PPNAD) (8, 55-57). More recently, mutations in the phosphodiesterase (PDE) genes *PDE11A* and *PDE8B* were found in isolated micronodular adrenocortical disease (iMAD) and iPPNAD, as well as in sporadic ADTs and other forms of BAHs (12, 34, 37).

However, the vast majority of benign ADTs do not bear germline or somatic sequence mutations in *GNAS*, *PRKARIA*, *PDE11A* or *PDE8B*. A minority of these tumors demonstrate abnormal expression of one or another of these genes due to somatic allelic losses or other, possibly epigenetic, events. For example, *PRKARIA* losses in sporadic ADTs and MMAD/ AIMAH without coding sequence alterations (58,59) or *PDE11A* under-expression in several ADTs, including CPAs, demonstrated by both mRNA and protein studies (34, 35, 36).

The cAMP signaling system can be roughly divided into the following compartments. At first, the generation of cAMP, secondly the response part (PKA enzyme) and finally the conclusion of the signaling event brought by the cAMP binding Phosphodiesterases.

The purpose of this study was to assess the overall activity of the cAMP-signaling pathway in ADTs that do not have coding sequence mutations or allelic losses for any of the above genes and genetic loci, respectively. These tumors were compared with those that had known *PRKAR1A* gene mutations and of course normal adrenal tissue. A relatively simple and general approach was taken: cAMP levels and cAMP binding affinity were assayed followed by total PKA and PDE activities along with the protein levels of the main molecular players. The data are somewhat surprising: they are suggestive of functional abnormalities of this pathway, albeit at different levels, in many of these lesions.

Moreover, we were interested in identifying the role of microRNAs in adrenal hyperplasias. The present study is the first one that attempts to investigate the role of microRNAs in any form of BAH; we selected MMAD/AIMAH and PPNAD as the first types of BAH to be studied for the role of microRNAs because although mutations in several genes have been considered as causative for BAHs, neither in MMAD/AIMAH nor in PPNAD the underlying pathogenetic mechanism is completely clarified. Also, because of the availability of a complete and recent set of expression data, and our access to several samples from patients with this disease.

2 Materials and Methods

2.1 cAMP signaling pathway studies

2.1.1 Subjects

Human studies were approved by the National Institutes of Child Health and Human Development under the protocol 95CH0059. Twenty seven patients (19 women and 8 men) , aged 5- 60 years with ADTs have participated in this study (Table 2.1).Tissue samples included thirteen from patients with MMAD, eight from patients with PPNAD and CNC carrying germline inactivating mutations of PRKAR1A (Table 2.2) and three patients with iMAD. Also, three samples from patients with cortisol producing adenomas were included in this study.

#	Patient code	SEX	Age(yr)	Diagnosis	Surgery	Dimensions		Weight		Diurnal cortisol levels		UFC
						Rt	Lt	Rt	Lt	pm	am	
1A	ADT03.01	M	54	MMAD/AIMAH	BADX	9x5x3	6x2.5x2	190	154	6.2	12.5	115.9
2A	Mt	F	49	MMAD/AIMAH	BADX	9x5x2	7x3x5	31.8	34.4	13.1	15.7	141.5
3A	ADT65.02	F	51	MMAD/AIMAH	UADX-right	6.2x4	-	111	-	7.9	10.2	18
4A	ADT55.02	F	42	MMAD/AIMAH	UADX-right	5	-	NA	-	3.2	8.2	8.2
5A	ADT36.01	M	46	MMAD/AIMAH	BADX	10x8x3.5	8x4.5x2.5	101	490	7.5	9.8	112
6A	CAR073.01	M	60	MMAD/AIMAH	BADX	12x8x4	14x7x4	155	182	12.8	16.2	179
7A	CAR589.01	M	39	MMAD/AIMAH	BADX	10x5.5x2.8	7x6x2.5	36	44	29.4	28.3	449
8A	ADT23.01	F	57	MMAD/AIMAH	BADX	8x8.5x3.5	11.5x5x2.5	65	54.3	22	31.1	191.6
9A	ADT29.03	M	45	MMAD/AIMAH	BADX	10.2x6.5x2.5	13x5x4	NA	131	12.6	19.9	132.7
10A	ADT27.03	F	43	MMAD/AIMAH	BADX	9x4x2	9x5x2	52.3	94.8	21.8	30.5	634
11A	ADT04.01	F	49	MMAD/AIMAH	BADX	3.5x3.5x2.8	2.3x1.8x1.5	NA	NA	32.3	30.6	242.2
12A	Mt01	F	46	MMAD/AIMAH	BADX	5x3x2.5	6.5x3x2.5	42.8	44.9	NA	NA	195.2
13A	ADT05.01	F	31	MMAD/AIMAH	UADX-right	5x2.5x2	-	22	-	22.4	21	278.6
1B	CAR021.02	F	23	CNC/PPNAD	BADX	10x2.1x1	7x3x1	NA	NA	11.4	11.8	194.3
2B	CAR599.02	F	41	CNC/PPNAD	BADX	NA	NA	NA	NA	13	16	200
3B	CAR616.02	F	30	CNC/PPNAD	BADX	7x6.5x1.5	10.5x4.5x2.5	43.4	51	13.5	14.2	61
4B	CAR047.01	F	35	CNC/PPNAD	BADX	4.5x2x1	5.5x4x1.5	17	12	8.7	13.7	4.6
5B	CAR053.03	F	22	CNC/PPNAD	BADX	5x3x1.8	5x2.5x2	NA	NA	32.2	31.2	176.3
6B	CAR020.14	M	13	CNC/PPNAD	BADX	4.5x3x0.5	5x3x0.5	13.25	13.4	3.8	8.8	25
7B	CAR79.03	M	5	CNC/PPNAD	BADX	3x1.3x0.8	3x1.5x.8	NA	NA	6.6	22	12
8B	CAR542.03	F	17	CNC/PPNAD	BADX	6x4x1	7x4x2.5	15	23	7.4	8.5	40
9B	CAR597.03	F	13	CNC/PPNAD	BADX	6.5x3x1.5	8.5x3.5x1.5	NA	NA	21.5	21.4	406
10B	CAR555.03	F	20	CNC/PPNAD	BADX	9x4.5x1.5	6x4.5x1.5	23	18	24.4	29.2	2261.6
11B	CAR583.03	F	10	iPPNAD	BADX	NA	NA	NA	NA	NA	NA	NA
1C	CAR77.03	M	19	iMAD	BADX	5x1x0.6	5x1x0.5	13.1	25.6	25	21.8	598
2C	CAR545.13	F	16	iMAD	UADX-left	-	7x3.5x1.5	-	15.6	5.2	7.1	44
3C	CAR653.02	F	30	iMAD	NA	NA	NA	NA	NA	NA	NA	NA
1D	ADT07.01	F	51	CPA	UADX-left	-	7x3.5x2.8	-	37	6	24	540.5
2D	CAR070.03	F	16	CPA	UADX-left	-	5.2x3.5x3.5	-	NA	19.6	19.5	444
3D	ADT54.03	F	18	CPA	UADX-left	-	5.5x4x3.5	-	23.67	22.9	18.4	181

Table 2.1: Patient clinical data

F, Female; M, Male; CS, Cushing Syndrome; MMAD, massive macronodular adrenocortical disease; CNC, Carney Complex; PPNAD, primary pigmented adrenocortical disease; CPA, cortisol producing adenoma; BADX, bilateral adrenalectomy; UADX, unilateral adrenalectomy; UFC, Urinary Free Cortisol; 17-OHS, 17-Hydroxy-steroid; Normal range: Diurnal Cortisol : 5-25 µg/dl; UFC:8-77 µg/24h; 17-OH – steroid: 3-10 mg/24h; Age(yr),age at the time of diagnosis; Patients 8-10B participated only in the microRNA studies.

Four normal adrenal tissues were collected during surgery from areas away of the adrenal lesion under investigation and were used as a control. Adrenal tissues collected during surgery were immediately dissected by the pathologist, periadrenal fat was removed and they were stored in liquid nitrogen until use.

2.1.2 cAMP levels assay

Levels of cAMP were measured in the tissue extracts using the cyclic AMP 3H Biotrak Assay System from Amersham Biosciences (Piscataway NJ). The tissue samples were homogenized in ethanol and centrifuged at 10.000 rpm for 10 min. The supernatant was dried and resuspended in 50μl of assay buffer according to the manufacturer's instructions. Each sample was processed in duplicates.

2.1.3 cAMP – binding protein assay

Gilman procedure has been used in order to measure the cAMP binding activity (60). Protein extract (see PKA activity below) 50μg in a total volume of 0.4 ml, containing 50mM of Tris 7.5, 8mM of $MgCl_2$ and 0.15 μM cyclic [3H] AMP was used as the incubation mixture. The samples were incubated for 50 min at 4°C and the reaction was stopped by adding 2ml of potassium phosphate buffer (20mM). The final mixture was passed through Millipore filters followed by washing with 15 ml of the same buffer (potassium phosphate buffer). The radioactivity was then assessed using liquid scintillation counting.

2.1.4 PKA activity

The activity of the PKA was measured following the protocol described earlier by Nesterova et al (61). Tissue samples were extracted in 10mM Tris-HCl pH7.5, 1mMEDTA, 0.1 mM DTT Protease Inhibitor cocktail I (EMDBiosciences, Darmstadt, Germany). About 10 ug protein of the tissue extracts were added to the reaction mixture (50 μl) containing 0.025 mM [γ -^{32}P] ATP, 5μM kemptide, 10 mM $MgCl_2$ plus or minus 5 μM cAMP and 5μM PKI. The mixtures were incubated for 15 min at 30°C, spotted on phosphocellulose filters and washed for three times using 0.1% phosphoric acid. The filters were left to air dry before analysis by liquid scintillation counter. PKA activity assay was performed in duplicates for each sample and data were analyzed using a two sample-t-test. A p value of less than 0.05 was considered

significant. Basal levels of PKA activity represent the non stimulated PKA activity and total PKA activity, reflects the PKA activity after the addition of cAMP. and Also, we calculated the PKA activity ratio; basal PKA activity/total PKA activity, to determine how much PKA is present in its active state.

2.1.5 PDE activity

A colorimetric method was used in order to determine the PDE activity in all our samples (BIOMOL Cyclic Nucleotide Phosphodiesterase, Plymouth Meeting, PA). This PDE assay is mainly based on the degradation of cAMP and cGMP by a cyclic nucleotide phosphodiesterase and the subsequent counting of the phosphate released, using the Biomol Green Reagent. Also, a non specific cyclic nucleotide phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) was used in order to study the degree of PDE inhibition in our samples.

2.1.6 Immunoblotting

Western blot analysis of proteins in the tissue extracts was performed. The extraction buffer was a mixture of 20mM Tris-HCl, PH 7.5, 5mM MgCl2, 1% NP40, 100mM NaCl, Protease Inhibitor Cocktail I and 0.5 % sodium deoxycholate. The lysates were centrifuged for 10 min, 10.000rpm, 4°C and the supernatant was collected. 10 μg of protein were subjected to SDS/PAGE (10% Tris-Glycine gel) and then transferred to 0.2μm nitrocellulose membranes. The membranes were probed with primary antibodies against PKA subunits: RIa (abcam), RIIa (Santa Cruz), RIIb and Ca (BD Bioscences). The blots were then washed and incubated with secondary antibodies against mouse or rabbit (1:1000; Amersham Biosciences) for 1 hour at room temperature. The bands were detected using enhanced chemiluminescence reagent (Amersham Biosciences). The images were quantified using the Image Quant Software and were normalized against the expression of β-actin (abcam).

2.1.7 Statistics

All assays were obtained in duplicate measurements and an average was calculated for each value. Comparisons were made using a two-sample t-test and p values were considered significant at $P<0.05$.

2.2 microRNAs studies

2.2.1 Subjects

The investigation of patients with CS caused by MMAD/AIMAH or PPNAD was completed under the National Institute of Child Health and Human Development protocols 95-CH-0059 and 00-CH-160. Both protocols were approved by the Institutional Review Board and all subjects signed informed consents. A total of 20 patients, aged 5-60 years, (table 2.1) were diagnosed with ACTH-independent CS that was caused by non-familial MMAD/AIMAH or PPNAD due to PRKAR1A inactivating mutations (table 2.2); the diagnosis was confirmed by histology post-operatively. Adrenal samples were collected during surgery and immediately dissected by the pathologist; periadrenal fat was carefully removed and all tissues were stored at -80^{o} C until further use.

#	Patient code	SEX	Age(yr)	Diagnosis	Mutations
1B	CAR 021.02	F	23	CS/CNC/PPNAD	c.101_105delCTATT/p.Ser34fsX9
2B	CAR599.02	F	41	CS/CNC/PPNAD	c.682C>T/p.Arg228X
3B	CAR 616.02	F	30	CS/CNC/PPNAD	c.491_492delTG/p.Val164fsX4
4B	CAR 047.01	F	35	CS/CNC/PPNAD	c.177+1G>A
5B	CAR 053.03	F	22	CS/CNC/PPNAD	c.43_58del16/p.Leu15fs 104X
6B	CAR 020.14	M	13	CS/CNC/PPNAD	c.491_492delTG/p.Val164fsX4
7B	CAR 079.03	M	5	CS/CNC/PPNAD	c.496C>T/p.Gln166X
8B	CAR 542.03	F	17	CS/CNC/PPNAD	c.279_282delTAGG/p.Arg94fsX34 c.2599C>G/p.P867G*
9B	CAR597.03	F	13	CS/CNC/PPNAD	c.865G>T/p.Gly289Trp
10B	CAR 555.03	F	20	CS/CNC/PPNAD	c.440+1 insG

Table 2.2: Patients with germline PRKAR1A mutations. (* additional mutation in PDE11A gene).

Four normal adrenal RNA samples were used as controls: two from two different healthy individuals (Ambion, Biochain) and another that was actually a pool of adrenal RNA from 5 mormal subjects (Biochain). Finally, we also used a sample from normal adrenal that was collected from a patient who was operated in our institution for an unrelated diagnosis and had to have adrenalectomy.

2.2.2 RNA extraction, microRNA array analysis and data validation

mirVana miRNA isolation Kit (Ambion, Inc, TX, USA) was used according to manufacturer's instructions in order to isolate total RNA. The quality of total RNA was tested using the Bioanalyzer 2100 and it was quantified using Nanodrop.

Expression levels of 365 microRNAs were evaluated with TaqMan microRNA array assays as previously described (62). Validation of these results was performed using the *mirvana* quantitative real time (qRT)-polymerase chain reaction (PCR) miRNA detection kit and qRT-PCR primer sets, according to the manufacturer's instructions (Ambion, Inc, TX, USA). The U6 small nuclear RNA was used as internal control, as suggested by others (62, 63).

Transcription of 0.1 ug RNA to cDNA was performed using the AMV kit (Roche, Indianapolis, USA). Lightcycler FastStart DNA master SYBR Green which contains Taq DNA polymerase, DNTPs mix, SYBR Green I dye and $MgCl_2$ was used as a reaction mix for PCR. GAPDH expression levels were used as a control. RT-PCR analysis was completed in the Light Cycler Instrument (Roche Molecular Systems, Alameda, CA). The oligonucleotide primers used for CPE have been described previously (63). The oligonucleotide primers used for WISP2 were 5'-CACGCTGCCTGGTCTGTCTGGATC-3' (forward) and 5'-CACGCATAGGCTTGTATTC AGGAAC-3' (reverse). All samples were analyzed in triplicate and the average value of the triplicates was used for quantification; data were expressed as the ratio of target gene mRNA levels *versus* those of the GAPDH housekeeping gene (62-66).

2.2.3 MicroRNA target prediction methods

We used three databases to detect the putative microRNA gene-targets: miRBase (http//microrna.sanger.ac.uk), Pictar (http://pictar.bio.nyu.edu) and Target scan version 4.2 (http://www.targetscan.org/ index.html) databases. Subsequently, we selected only the commonly predicted microRNA targets from the three databases and those that were also conserved in other species, aiming to the higher biological significance of our results. The data from this combined prediction analysis were compared with the cDNA array data (17) for the MMAD and SAGE data presented in our previous study (10) for the PPNAD. Knowing that microRNAs most commonly act by suppressing the expression of their target genes, we studied only the microRNA -gene target pairs with inversed correlation.

2.2.4 Cell line characterizations

We used a cell line that was derived from the adrenocortical tissue of patient CAR 047.01 (Table 2.1, 2.2), as previously described (67). The cell line has been frozen

since and it grows without any changes. According to our recent study on this particular cell line the PKA system remains hyperactivated. Specifically, introduction of the wt R1A subunit in these cells resulted in growth inhibition as well as in decreased cAMP levels compared to those of the parental cells indicating the malfunction of the PKA system (68).

2.2.5 microRNA transfection experiments

PPNAD cells were seeded in 6-well plates and were transfected with 20 or 50nM miR-449 (Ambion, Inc, TX, USA) using siPORT *NeoFX* transfection kit. siPORT *NeoFX* is a lipid transfection agent consisting of a mixture of lipid that spontaneously complex microRNAs and facilitates its transfer to the PPNAD cells. Transfection with 50nM of scramble negative control microRNA was used as an internal control. No cell toxicity was detected due to the transfection agent. RNA was extracted 24 and 48 hours after microRNA transfection and Real Time PCR analysis was performed as described above.

2.2.6 PKA inhibitor (H89) treatment

PPNAD cells were treated with 1uM H89 for 2h and miR-449 expression was evaluated by real-time PCR analysis. Furthermore, WISP2 mRNA expression was evaluated 24h after H89 treatment by real-time PCR.

2.2.7 Luciferase reporter assay

293 cells in 24-well plates were transfected using Fugene6 (Roche, Penzberg, Germany). Firefly luciferase reporter gene construct (WISP2 3'UTR in pEZX-MT01 vector) (200 ng) and 1 ng of the pRL-SV40 Renilla luciferase construct (for normalization) were co-transfected per well. Cell extracts were prepared 24h after transfection, and the luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

3 Results

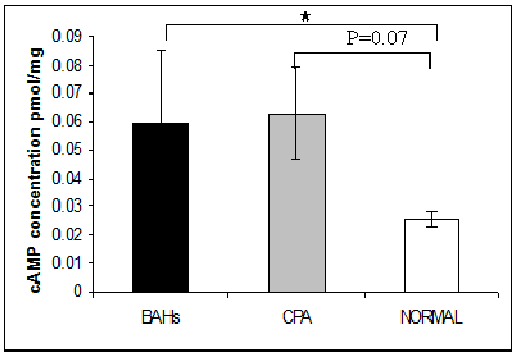
3.1 cAMP signaling pathway studies

3.1.1 Genetic studies

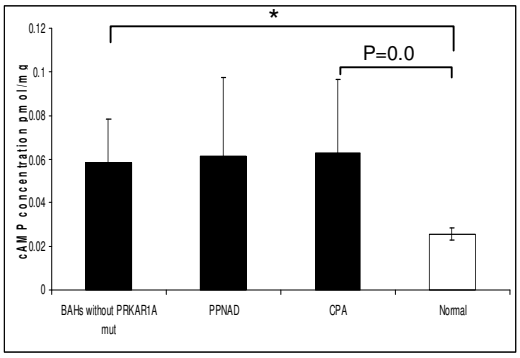
A total of 27 patients with ADTs causing ACTH-independent CS were studied (Table 2.1); both tumor and peripheral DNA was studied for *GNAS*, *PRKARIA*, *PDE11A* and *PDE8B* gene mutations and allelic losses of the respective chromosomal loci (data not shown). Only the PPNAD patients had germline mutations of the *PRKAR1A* gene; these mutations are listed in Table 2.2.

3.1.2 cAMP levels and cAMP binding affinity

All ADTs had higher cAMP levels than normal adrenal tissue (figure 3.1). When subgrouped, CPAs (0.063 $\pm$ 0.034 pmol/mg) were not different from MMAD (0.063 $\pm$ 0.015 pmol/mg) or PPNAD caused by *PRKAR1A* mutations (0.061 $\pm$ 0.036 pmol/mg). In addition, all cortisol-producing ADTs (with the exception of PPNAD) had higher cAMP binding affinity than normal adrenal tissue (12.6 +/- 4.07 cpm/µg) (figure 3.2). CPAs had the highest cAMP binding affinity (34.1 $\pm$ 8.307 cpm/µg) (*P*=0.006) followed by MMAD (26.2 $\pm$ 13.9 cpm/µg) (*P*=0.08). PPNAD, a disease that is caused by inactivating mutations of the most important endocellular cAMP receptor, *PRKAR1A*, had the lowest cAMP binding affinity (9.78 $\pm$ 7.8 cpm/µg) (figure 3.2).

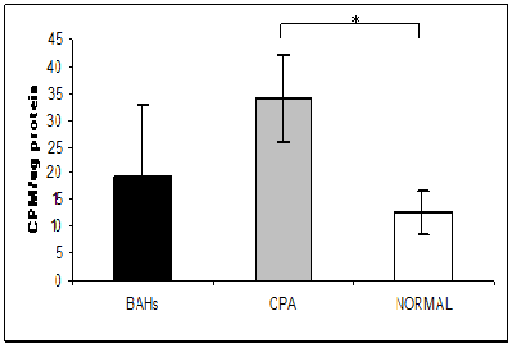


a

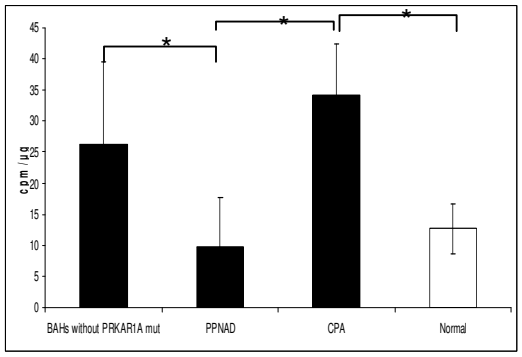


b

Figure 3.1: a, Levels of cAMP in BAHS, CPA and normal adrenal; b, Levels of cAMP in BAHs without PRKAR1A mutations (MMAD, iMAD), PPNAD and CPA.



a

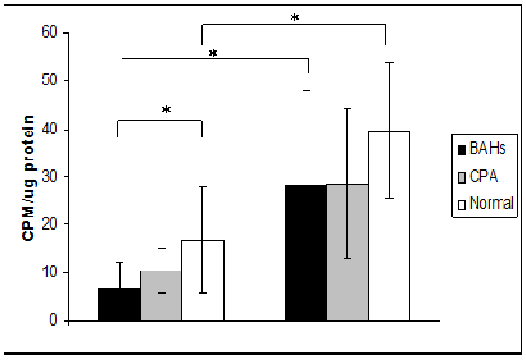


b

Figure 3.2: a, cAMP binding affinity in BAHS, CPA and normal adrenal; b, cAMP binding affinity in BAHs without PRKAR1A mutations (MMAD, iMAD), PPNAD and CPA.

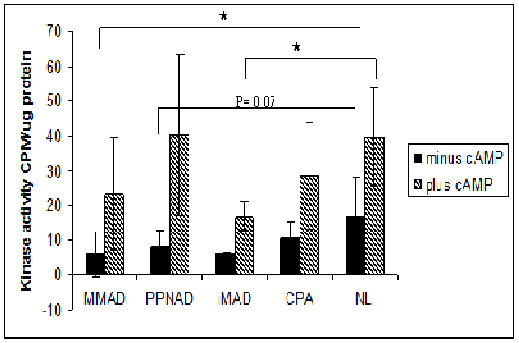
3.1.3 PKA assay

Kinase activity was determined with and without the addition of cAMP and PKI in all samples. All adrenal hyperplasias had statistically significant lower basal PKA levels than normal adrenal (6.73 ± 5.4 cpm/µg) (P=0.0059); there were no statistically significant differences between individual diagnostic subgroups (figure 3.3). Upon stimulation with cAMP, there were no significant differences (*P*>0.05): normal adrenal gland: 39.7 +/- 1.4 cpm/µg, CPA: 28.5 ± 15.4 cpm/µg, iMAD 16.9 ± 4.2 cpm/µg; MMAD: 23.3 ± 16.3 cpm/µg, and PPNAD: 40.4 ± 23.2 cpm/µg. Adrenal hyperplasias had lower free PKA activity than normal adrenal (16.8 ± 10.9 cpm/µg) (*P*= 0.0045) and among them, PPNAD had the lowest (7.9 ± 4.3 cpm/µg of protein). Overall, adrenal hyperplasias had significantly lower PKA activity ratio (figure 3.4) in comparison to normal adrenal (0.24 ± 0.11 cpm/µg vs. 0.41 ± 0.14 cpm/µg, respectively, *P*=0.01), and adenomas (0.38 ± 0.04 cpm/µg, *P*=0.043). Within BAHs, PPNAD had lower PKA activity ratio than iMAD (*P*=0.033)



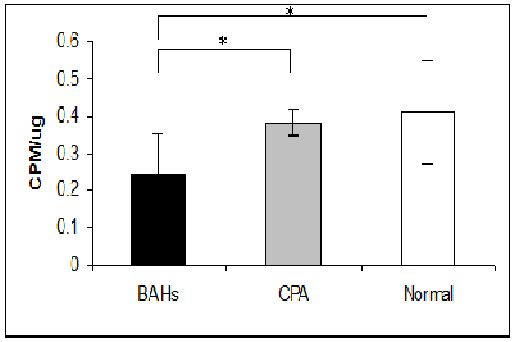
a

cAMP - +

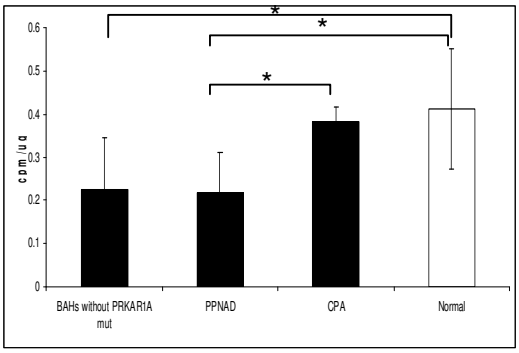


b

Figure 3.3: a, Basal and total PKA activity in BAHS, CPA and normal adrenal; b, Basal and total PKA activity in the subgroups of BAHs (MMAD, PPNAD, iMAD).



a

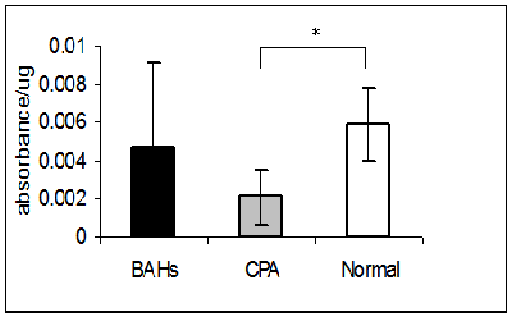


b

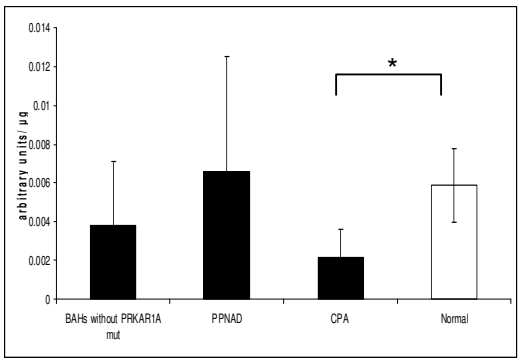
Figure 3.4: a, PKA activity ratio in BAHS, CPA and normal adrenal; b, PKA activity ratio in BAHs without PRKAR1A mutations (MMAD, iMAD), PPNAD and CPA.

PDE activity

Adenomas had the lowest PDE activity compared to normal adrenal (0.0021 $\pm$ 0.014 vs. 0.0058 $\pm$ 0.002 arbitrary units/µg, P=0.037) (figure 3.5). Adrenal hyperplasias as a group (0.047 $\pm$ 0.004 arbitrary units/µg) had no different PDE activity from normal tissue (P>0.1); individual diagnostic groups were not different from each other, although MMAD was closer to adenomas and tended to be different from normal adrenal (P=0.1).



a



b

Figure 3.5: A, PDE activity in BAHS, CPA and normal adrenal; B, PDE activity in the subgroups of BAHs (MMAD, PPNAD, iMAD).

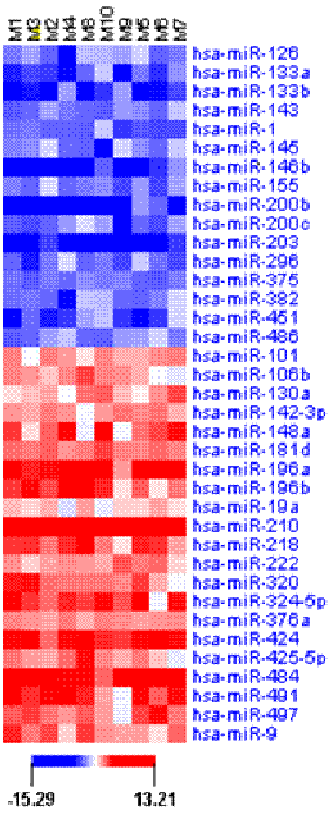
3.1.4 Western Bloting

We have performed Western Blotting against the main subunits of PKA in order to detect differences in the expression of these proteins. According to our findings, no significant difference was found in the expression of PKA subunits among the ADTs and the normal adrenal tissues (Data not presented here).

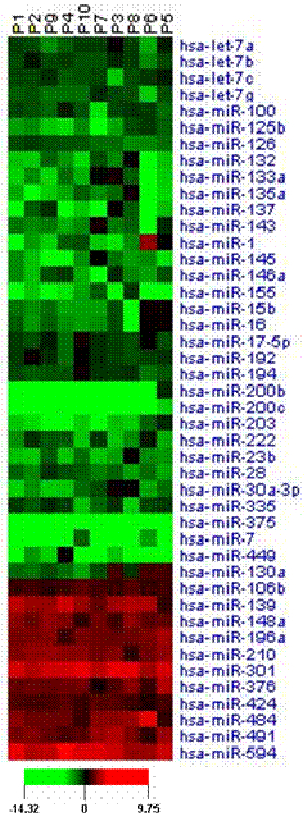
3.2 microRNA studies

3.2.1 miRNA gene signature in MMAD/AIMAH and PPNAD

We tested the expression of a total of 365 microRNAs and identified 37 differentially expressed microRNAs between MMAD/AIMAH and normal adrenal RNA and 44 microRNAs which had aberrant expression between PPNAD and normal adrenal. Specifically, in MMAD/AIMAH samples we detected 16 down-regulated microRNAs and 21 up-regulated ones. Down-regulated microRNAs in MMAD/AIMAH included miR-200b, miR-203, miR-133a and -133b, whereas up-regulated microRNAs were miR-210, miR-484, miR-148a and miR-130a. In PPNAD, 33 microRNAs were under-expressed and 11 were over-expressed in comparison to normal samples. Examples of down-regulated microRNAs in PPNADs were the let-7 family (let-7a, let-7b, let-7c,let-7g) and the miR-200 family (miR-200b, miR-200c). Among the up-regulated ones we detected miR-301, miR-210 and miR-106b. These data are presented as a heatmap after hierarchical clustering in figure 3.6 a, b. Several microRNAs were commonly up- or down-regulated in both MMAD/AIMAH and PPNAD (figure 3c).



a



b

down-regulated	up-regulated
miR-106b	miR-126
miR-148a	miR-133q
miR-196a	miR-143
miR-210	miR-1
miR-376	miR-145
miR-424	miR-155
miR-484	miR-200b
miR-491	miR-200c
	miR-203
	miR-375

c

Figure 3.6: Heat map representation of the expression pattern for 365 microRNAs studies in a, 10 MMAD/AIMAH samples; b, 10 PPNAD samples and 4 normal adrenals; c, MicroRNAs that were found up- or down regulated in both MMAD, PPNAD.

A color code is used for the level of microRNA expression: blue, green: lower microRNA expression, red: higher microRNA expression.

3.2.2 Validation of microRNA microarray data

SYBR Green qRT-PCR analysis validated the microRNA gene signature set that clearly distinguished MMAD/AIMAH and PPNAD tissues from normal adrenal samples (Figure 9A,B). The microRNAs that were most highly down-regulated in MMAD/AIMAH included miR-200b (- 9.36 fold), miR-203 (-7.68 fold) and miR-146b (-6.33fold). Other down-regulated microRNAs were decreased from -4.05 to -1.74 fold. Highly upregulated microRNAs in MMAD/AIMAH were miR-210 (10.78 fold), miR-484 (6.69 fold), miR-196a (5.66 fold) and miR-424 (5.23 fold); other upregulated microRNAs increased from 4.42 to 1.48 fold (figure 3.7a). As far as it concerns PPNAD, 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-7 (-7.33 fold); the rest of the down-regulated microRNAs had a decrease of 1.62 to 4.5 fold. MiR-594 (7.38 fold), miR-301 (7.57 fold) and miR-139 (5.33 fold) were the most highly up-regulated ones in PPNAD and the other microRNAs with increased expression had an increase of 1.84 to 3.94 fold (figure 3.7b).

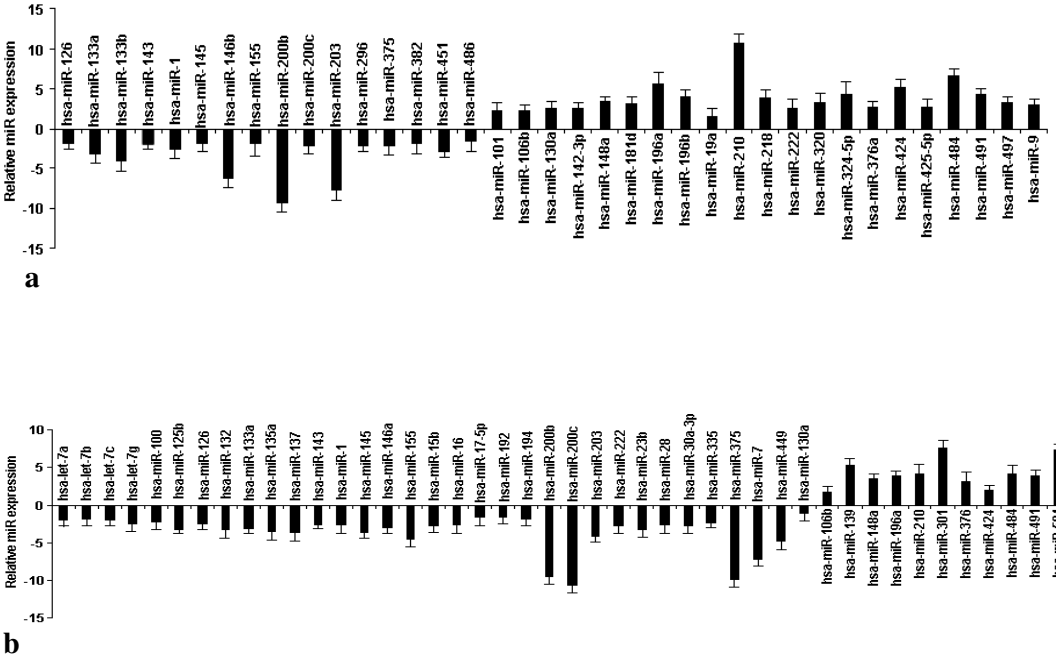


Figure 3.7: Real Time PCR of the microRNAs differentially expressed between a, MMAD; b, PPNAD and normal adrenals according to the microarrays,that confirmed the array results.

3.2.3 Detection of microRNA gene targets

As described in methods, we used the TargetScan microRNA target prediction algorithm that can identify potential gene targets for microRNAs. To identify microRNAs modulating important signaling pathways in MMAD/AIMAH, we integrated the microRNAs-gene target pairs predicted by Target Scan with the cDNA microarray data from our previous study (17). We identified 20 microRNA-gene target pairs that could be involved in macronodular adrenal hyperplasia (Supplemental figure 0.1). Table 3a lists the microRNAs, their chromosomal location and predicted target genes.

Table 3 . MicroRNA – gene target pairs in A, MMAD ; B, PPNAD

MicroRNA gene	Chromosomal location[1]	Putative Targets[2]	Description
miR-145	5 : 148790402-148790489	TARS	Threonyl-tRNA synthetase
miR-146b	10 : 104186259-104186331	PSMC6	Proteasome (prosome, macropain) 26S subunit, ATPase, 6
		PHKB	Phosphorylase kinase, beta
miR-155	21 : 25868163-25868227	HIF1A	Hypoxia-inducible factor 1 alpha
miR-200b	1 : 1092347-1092441	MATR3	Matrin 3
		CTSO	Cathepsin O
		PUM2	Pumilio homolog 2 (Drosophila)
miR-200c	12 : 6943123-6943190	MATR3	Matrin 3
		CTSO	Cathepsin O
		PUM2	Pumilio homolog 2 (Drosophila)
miR-203	14 : 103653495-103653604	CSPG2	Versican
		MATR3	Matrin 3
		TLOC1	Translocation protein 1
		CCNG1	Cyclin G1
		KRT1	Keratin 1
		PUM2	Pumilio homolog 2 (Drosophila)
miR-382	16 : 65793725-65793799	PRG4	Proteoglycan 4
miR-148a	3 : 189869263-189869348	TXNIP	Thioredoxin interacting protein
miR-196a	7 : 27175624-27175707	CPE	Carboxypeptidase E
miR-196b	12 : 52671789-52671898	CPE	Carboxypeptidase E

[1]MicroRNA chromosomal location (human genome) according to the miRBase database from Sanger Institute.

[2]Putative microRNA targets fullfilling the criteria described in Supplementary Figure 1.

Examples of predicted microRNA – target genes are miR-145 and Threonyl-tRNA synthetase *(TARS)*, miR-146b and Proteasome (prosome, macropain) 26S subunit ATPase 6 *(PSMC6)*, miR-210 and Phosphorylase kinase beta *(PHKB)* and Hypoxia-inducible factor 1 alpha (*HIF1A*). Also, miR-200b and -200c target Matrin 3

(*MATR3)*, Cathepsin O *(CTSO)*, and Pumilio homolog 2 *(PUM2)*. *PUM2*, *MATR3* along with Versican *(CSPG2)*, Translocation protein 1 *(TLOC1)*, Cyclin G1 *(CCNG1)* and Keratin 1 (*KRT1)* are gene targets of Mir-203; the remaining pairs were miR-382 and Proteoglycan 4 *(PRG4)*, miR-148a and Thioredoxin interacting protein *(TXNIP)* and miR-196a, -196b and carboxypeptidase E *(CPE).* For validation of the above, we did test the expression of the miR-196a and CPE with qRT-PCR and confirmed the inverse correlation of the microRNA and the target gene expression in our patients (R= -0.992) (figure 3.8).

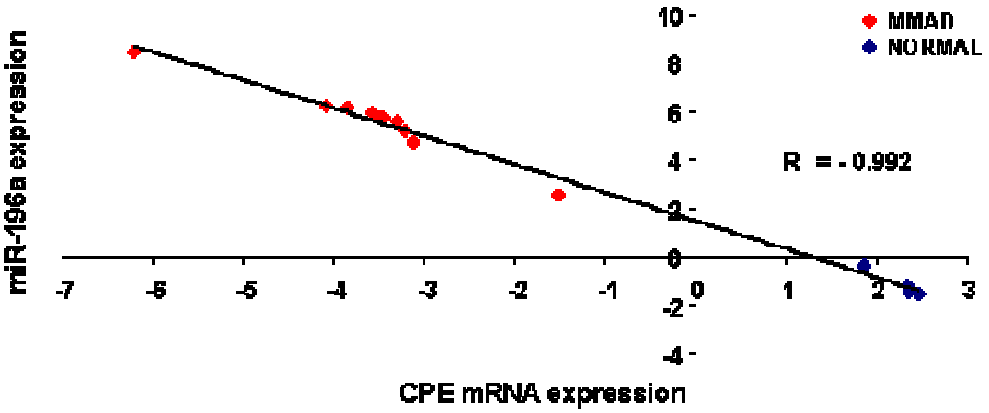


Figure 3.8: Confirmation of the inverse correlation of miR-196a and the target gene carboxypeptidase E (CPE) with Real Time PCR in MMAD patients and normal subjects (R=-0.092).

For the PPNAD tissues, we integrated microRNA and SAGE data together (10) with the bioinformatic prediction algorithms in order to identify microRNA gene-target pairs that would act as regulators in adrenals tumorigenesis (figure 3.9).

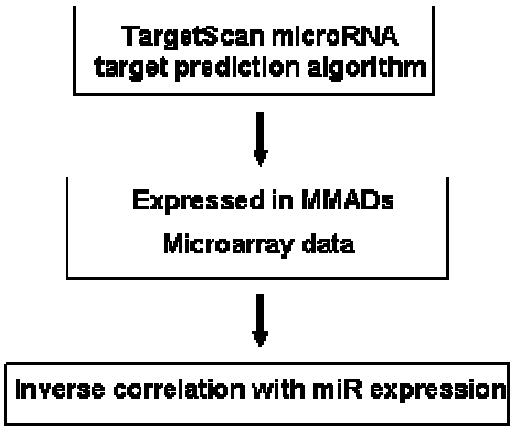


Figure 3.9: Detection of microRNA – gene targets with biological significance.
We used the microRNA target scan prediction algorithm and we identified the potential gene targets for the differentially expressed microRNAs between MMADs/ PPNADs and normal adrenal tissues. Subsequently, we integrated the results with expression data on MMAD (17) and on PPNAD (10) and finally we focused on the genes that had inverse correlation with the microRNA expression.

Again, we selected only the gene targets that were inversely correlated with microRNA expression, in order to have biological significance (Suppl. Figure 0.2). In table 3b, we present the 22 microRNA – gene targets that are presumably implicated in PPNAD pathogenesis. Several microRNAs target more than one genes and more importantly, few genes such as Nephroblastoma overexpressed gene (*NOV*), Cytochrome P450, family 11, subfamily B, polypeptide 1 (*CYP11B1*) and FBJ murine osteosarcoma viral oncogene homolog B (FOSB) are paired with more that one microRNA, indicating their important role in adrenal tumorigenesis. Specifically, we found that miR-203 targets *NOV*, dopamine beta-hydroxylase (*DBH*), insulin-like growth factor binding protein 5 (*IGFBP5*) and *FOSB*. *FOSB* is also a target for miR-23b and miR-1. Additionally, miR-200b, miR-200c and miR-23b target *CYP11B*. MiR-200c along with mir-132 and miR-203 are pairs with *NOV*. Inhibin alpha (*INHA*) is the gene target of miR-7 and regulator of G-protein signaling 5 (*RGS5*) can be targeted by miR-23b. Serine/threonine kinase 19 (*STK19*) and cyclin D2 (*CCND2*) are both potential targets of miR-15b and miR-16; chromogranin A (parathyroid secretory protein 1) (*CHGA*) is also targeted by mR-15b. Other predicted pairs included miR-17-5p and steroidogenic acute regulatory protein (*STAR*), let-7g and cytochrome

P450, family 21, subfamily A, polypeptide 2 (*CYP21A2*) and miR-28 and G protein-coupled receptor 107 (GPR107). Finally, miR-449 targets WNT1 inducible signaling pathway protein 2 (WISP2). In order to evaluate our predicted gene target- microRNA pairs we performed Real Time PCR for the miR-449- WISP2 pair and we confirmed its inverse correlation in the PPNAD samples (figure 3.12a).

MicroRNA gene	Chromosomal location[1]	Putative Targets[2]	Description
miR-203	14 : 103653435-103655604	NOV	Nephroblastoma overexpressed gene
		DD	Dopamine beta-hydroxylase
		FOSB	FBJ murine osteosarcoma viral oncogene homolog B
		IGFBP5	Insulin-like growth factor binding protein 5
miR-200b	1 : 1092947-1093247	CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1
miR-200c	12 : 6943123-6943190	NOV	Nephroblastoma overexpressed gene
		CYP11D1	Cytochrome P450, family 11, subfamily D, polypeptide 1
miR-132	17 : 1886952-1887052	NOV	Nephroblastoma overexpressed gene
miR-7	9 : 85774483-85774592	INHA	Inhibin, alpha
miR-16b	3 : 161605270-161605167	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-23b	9 : 98887317-98887407	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
		CYP11D1	Cytochrome P450, family 11, subfamily D, polypeptide 1
		RGS5	Regulator of G-protein signaling 5
miR-17-5p	13 : 90800863-90800943	StAR	Steroidogenic acute regulatory protein
miR-1	18 : 17892987-17893047	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
let-7g	6 : 52277357-52277417	CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2
miR-28	3 : 169889263-169889316	GPR107	G protein-coupled receptor 107
miR-449	5 : 57602117-57602207	WISP2	WNT1 inducible signaling pathway protein 2

[1]MicroRNA chromosomal location (human genome) according to the miRBase database from Sanger Institute

[2]Putative miRNA targets fulfilling the criteria described in the fig 3

3.2.4 Correlation of microRNAs and clinical data

As a test of the clinical significance of the above data, we sub-grouped our MMAD/AIMAH patients in 3 categories based on the "severity" of their disease (Table 3A): group a (patients 4, 1, 5, 3) included patients with midnight cortisol levels less than 10mcg/dl; group b (patients 9, 6, 2) had cortisol levels that ranged between 10-20mcg/dl, and group c (patients 10, 8, 7) over 20mcg/dl. Upregulation of miR-130a (r =0.91) and miR-382 (r =0.86) were associated with higher midnight cortisol levels (figure 3.10).

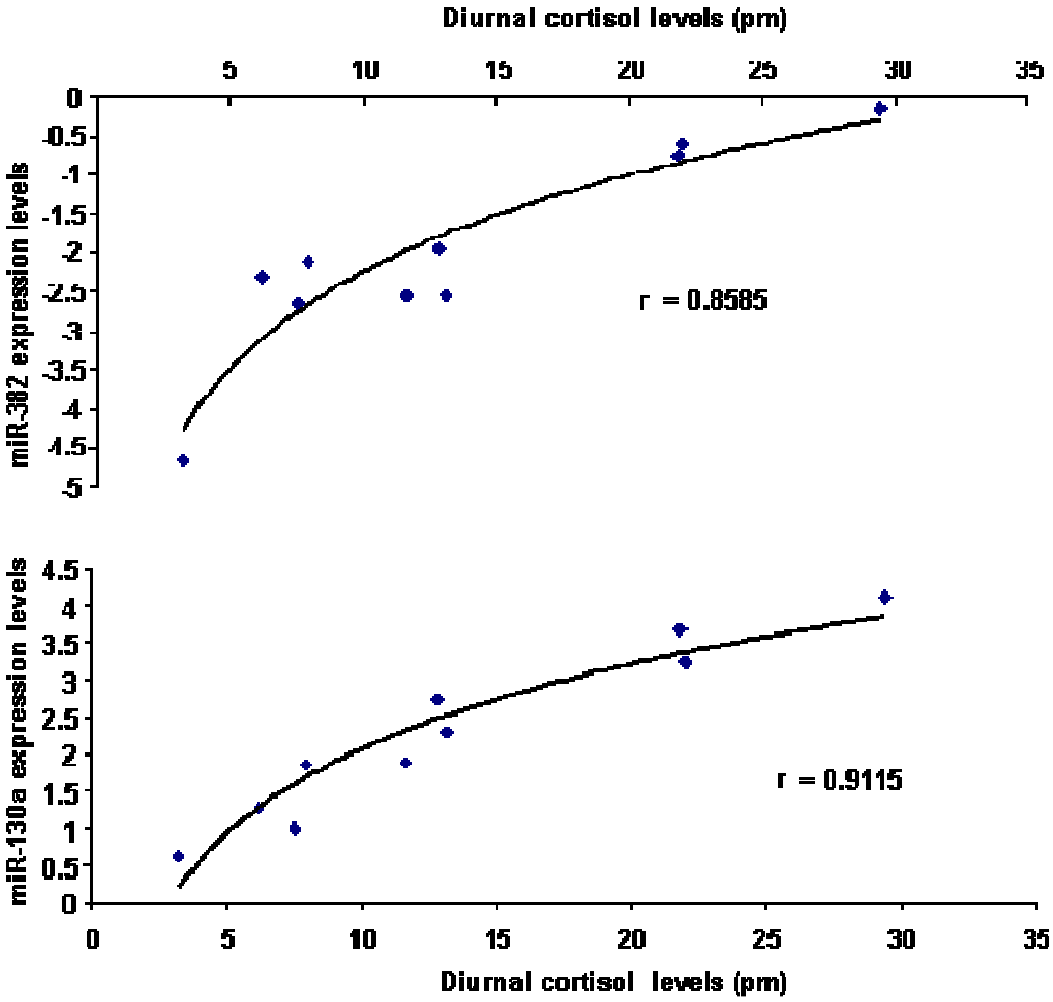


Figure 3.10: Micro-RNAs miR-382 and miR130a expression have a positive correlation with diurnal cortisol levels in MMAD patients.

Regarding the PPNAD patients, again in the first group we included those with diurnal midnight cortisol levels < 10μg/dl (patients 1-4), the second group had cortisol levels between 10-20 μg/dl (patients 5-7) and in the third one cortisol was >20μg/dl (patients 8-10). Comparison between microRNA expression levels and diurnal midnight cortisol levels revealed that let-7b expression was highly correlated to cortisol levels (figure 3.11). Specifically let-7b expression was inversely correlated (r=-0.9499) with diurnal midnight cortisol levels.

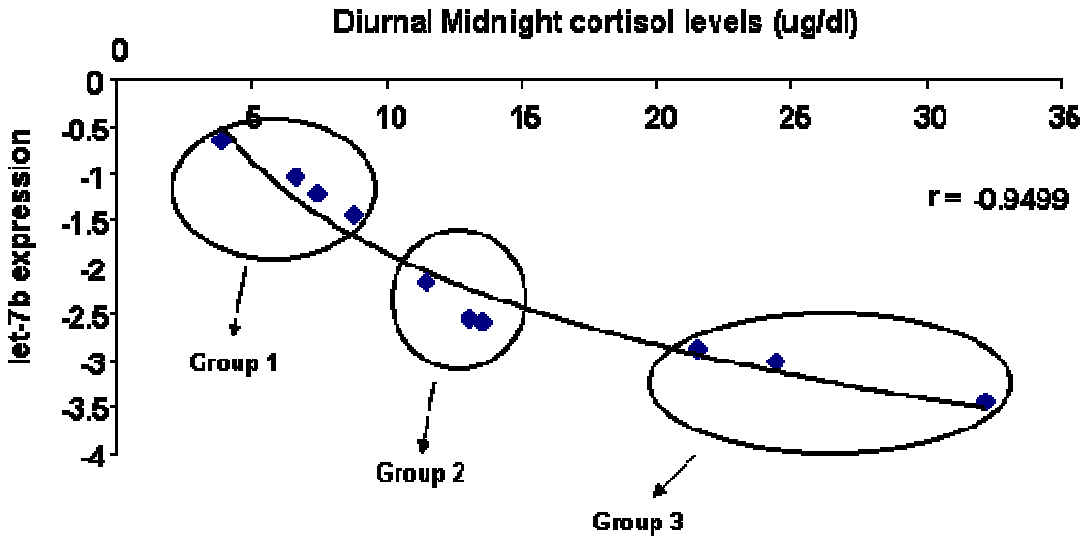


Figure 3.11: Correlation of let7b expression and midnight cortisol levels.

3.2.5 MiR-449 regulates WISP2 expression in PPNAD.

One of the most important pathways that seems to be involved in PPNAD tumorigenesis is Wnt-signaling pathway. Wnt pathway is essential in adrenocortical development (69); additionally somatic mutations have been found in adrenocortical adenomas and carcinomas (40). In the present study we found that *(WISP2)* Wnt induced secreted protein 2 *(WISP2)* is upregulated in PPNAD patients relative to controls according to the SAGE data. In order to study in greater detail the role of WISP2 in PPNAD we performed Real time PCR analysis. We detected that WISP2 is 2.32 fold upregulated in PPNAD in comparison to normal, confirming that Wnt pathway is activated in PPNAD (figure 3.12a). According to our integrative analysis we found that miR-449 targets WISP2 (Table 3B, Supplemental figure 0.2). Evaluation of miR-449 expression by real-time PCR revealed that is 4.83-fold down-regulated in PPNAD samples in comparison to normal samples (figure 3.12a). The inverse correlation between miR-449 and WISP2 mRNA expression levels suggested that miR-449 regulates Wnt pathway in PPNAD pathogenesis. In order to test the potential interaction between miR-449 and WISP2, we transfected miR-449 in a PPNAD cell line and examined WISP2 expression. We identified that miR-449 overexpression (50 nM) suppressed >70% WISP2 mRNA expression (figure 3.12b). Next we sought to identify if miR-449 regulated directly WISP2 through binding in the 3'UTR. Luciferase assay revealed that miR-449 inhibited >60% WISP2 3'UTR

luciferase activity, suggesting that miR-449 regulates directly WISP2 expression (figure 3.12c).

3.2.6 PKA activates Wnt pathway through miR-449 inhibition.

PKA signaling pathway is shown to be activated in PPNAD cell line which bear inactivating mutations of PRKARIA mainly because of upregulation of other PKA subunits, such as PRKARIIB (68). In these cells, miR-449 was found downregulated while WISP2 was highly expressed. According to our microRNA microarray data from PPNAD patients, miR-449 was 4.8 fold down-regulated. Overexpression of miR-449 in PPNAD cell line resulted in inhibition of WISP2 expression, suggesting that miR-449 targets WISP2. PPNAD samples have increased PKA activity and our microRNA microarray analysis revealed that miR-449 is highly down-regulated in these tissues. In order to detect if miR-449 is regulated by PKA, we treated PPNAD cells with H89 (1uM) and tested miR-449 expression. We found that inhibition of PKA activity increases miR-449 expression (figure 3.12d). In addition, the increase miR-449 expression is followed by a decrease in WISP2 expression levels (figure 3.12e). All these results suggest that PKA activation blocks miR-449 expression and miR-449 inhibition allows up-regulation of its target gene WISP2.

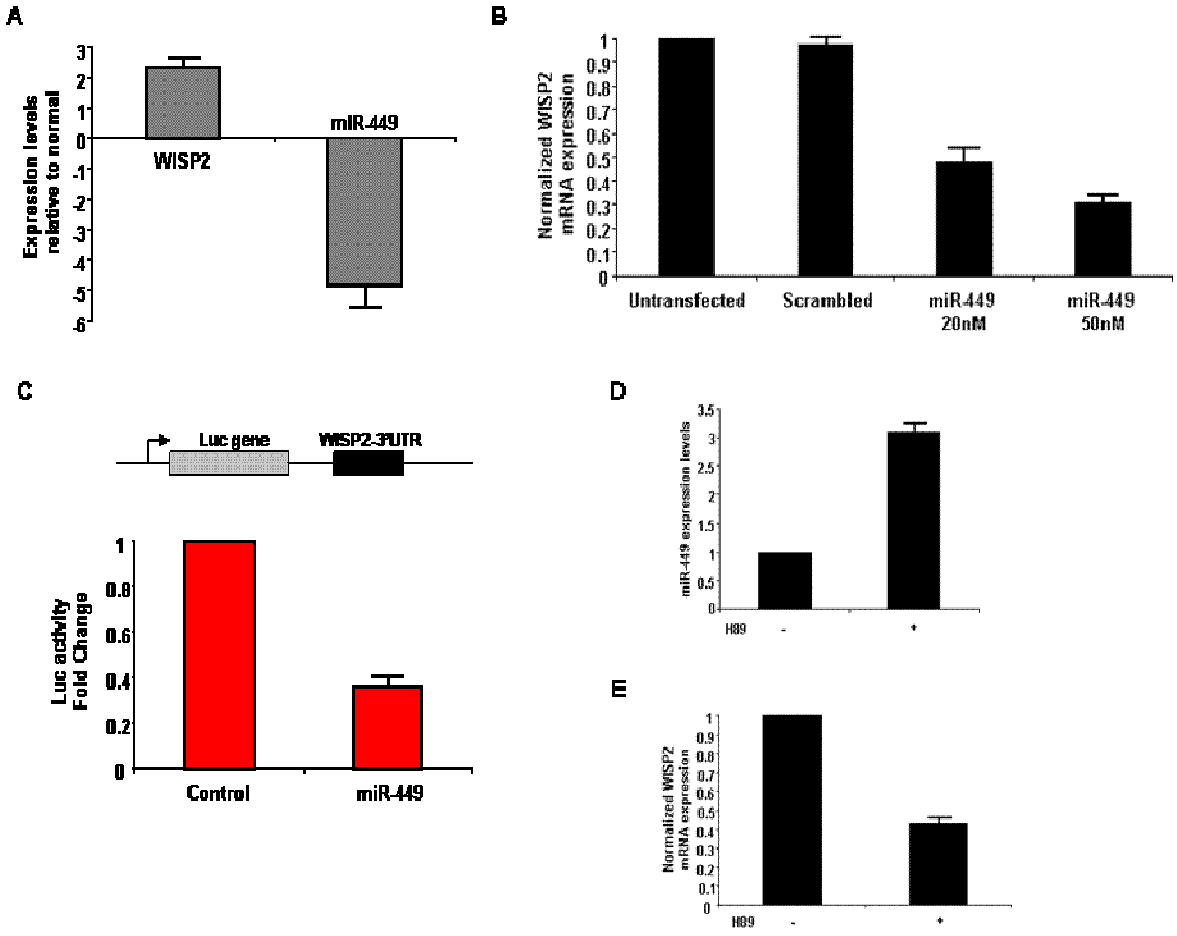


Figure 3.12: PKA regulates WNT signaling through miR-449.

(a) Confirmation with RT-PCR that WISP2 is overexpressd and miR-449 is down-regulated in PPNAD. (b) Transfection of miR-449 in our PPNAD cell line (20nM, 50nM) reduced WISP2 expression (50% and >70% suppression of mRNA expression respectively). (c) Mir-449 binds to the 3'UTR of WISP2 gene, inhibiting more than 60% of WISP2 3'UTR luciferase activity.(d) Inhibition of PKA activity with H89 inhibitor increases the expression of miR-449 (e) and decreases the expression of WISP2.

4 Discussion

4.1 cAMP signaling pathway studies

In the present study, we investigated cAMP levels, binding affinity and PKA and PDE activities in ADTs that had no mutations in the *GNAS*, *PRKARIA*, *PDE11A* and *PDE8B* genes and compared these data with those derived from samples with PPNAD and *PRKARIA* mutations, and a limited number of normal adrenal glands. The data show that these benign tumors have abnormalities in the cAMP pathway; for example all ADTs tested had higher cAMP levels than normal adrenal tissue. Likewise, cAMP-binding affinity was higher than normal adrenal tissue in all non-*PRKAR1A*-related lesions, independently of the histology (CPA, MMAD/AIMAH, iMAD). PPNAD that is due to inactivating *PRKAR1A* mutations (8,55-56) was expected to have low cAMP-binding affinity.

These tumors were not tested for aberrant expression of G-protein coupled receptors (GPCR) but none of these lesions bore any of the known mutations of the cAMP pathway. Although aberrant GPCR expression could explain the higher cAMP levels (51, 53) in at least MMAD/AIMAH, cAMP binding affinity should not be affected by this phenomenon (68). Alterations of cAMP endocellular receptors are likely, as well as changes in the kinetics of cAMP responsiveness, are probably responsible for this observation.

PDE activity was measured for the first time in cortisol-producing ADTs; our data indicated a marked decrease in PDE activity in CPAs and a non-significant decrease in MMAD. These were lesions that did not have mutations in the known PDE genes that are mutated in ADTs (*PDE11A* and *PDE8B*), indicating that, perhaps, additional PDEs are involved in the genesis of benign adrenocortical lesions. In fact, one of the reasons that we had so few adenomas in this study, was that we had very few that did not have even benign polymorphisms of the *PDE11A* gene. *PDE11A* gene variants are very frequent in ADTs (26, 35), but the present data suggest that additional PDEs may be involved.

Furthermore, the PKA activity ratio was decreased in BAHs compared to normal tissue and single adenomas. This finding was expected for samples bearing *PRKAR1A* mutations but not for samples from other BAHs, such as iMAD and MMAD/AIMAH that did not contain *PRKAR1A* or 17q22-24 defects (59). PKA activity ratio indicates the amount of PKA that is in its active form (8, 60,61,68). These data suggest that functional abnormalities of the PKA enzyme may be present in BAHs even when *PRKAR1A* and its chromocomal locus are not involved.

4.2 microRNAs

The present study, the first of its kind, identified 37 microRNAs which were statistically different in terms of their expression in patients with MMAD/AIMAH in comparison to normal adrenal tissue and 44 microRNAs in PPNAD in comparison to normal tissue. 10 microRNAs were down-regulated and 8 were up-regulated in both MMAD/AIMAH and PPNAD.

In both MMAD/AIMAH and PPNADs several microRNAs were downregulated. miR-200b and miR-200c are down-regulated in metastasis and have an important role in epithelial-mesenchymal transition (70) although they are over-expressed in human ovarian cancer (71). MiR-203 is downregulated in several types of cancer, including oral squamous cell carcinoma (72); MiR-203 may act as a tumor suppressor and its putative target gene is *ABL1*, a well known oncogene that is activated in chronic myelogenous leukemia (73). MiR-203 also targets the transcription factor p63 involved in proliferation and differentiation of epithelial cells (74). miR-155 is regulated by the NF-kB signaling pathway(75), as well as by TGF-β through Smad 4 and influences cell migration in cancer cell metastasis (76). MiR-145 is down-regulated in colorectal cancer; *MAPK3* and the protooncogene *YES1* are included among its putative targets (77). Mir-1 expression is also reduced in human lung and hepatocellular carcinomas and its ectopic expression suppressed cell proliferation and was associated with down-regulation of the levels of *FoxP1* and *MET* (78, 79). MiR-375 is expressed in pancreatic beta cells and is involved in the PI3-kinase signaling pathway by regulating the expression of 3-phosphoinositide-dependent protein kinase-

1 (*PDK1)* (80); miR-126 down-regulation induces cell proliferation by activation of PI3K-AKT and ERK1/2 signaling pathways (81).

In MMADs, we found reduced expression of several microRNAs (16/37) which have been also shown to be altered in various cancers: MiR-146b and miR-222 are both upregulated in papillary thyroid carcinoma; miR-146b in particular is suggested as a diagnostic marker for this type of cancer (82). MiR-146b is involved in innate immune responses mediated by cytokines and the Toll-like receptor by regulating the expression of TNF receptor-associated factor 6 and IL-1 receptor (*IL1R*)-associated kinase 1 (75). IL1R has been implicated in Cushing syndrome caused by adrenocortical adenomas (83). miR-133b is also under-expressed in colorectal cancer and it also targets *MAPK3* and *YES1* as miR-145; additionally, *KRAS* is predicted to be another target of miR-133b (77). MiR-133*a* and -*b* are also down-regulated in MMAD/AIMAH and appear to be involved in the regulation of pyruvate kinase type M2 (*PKM2)* in head and neck cancer (84). MiR-451 transcription is modulated by the hematopoietic transcription factor GATA-1, and is required for normal erythropoiesis by regulating its target GATA-2, according to studies in zebrafish embryos (85, 86). Moreover, miR-451 is involved in the regulation of multiple drug resistance mediated by P-glycoprotein (the *MDR1* gene) in cancer cells (87).

In PPNADs, we found 33 down-regulated microRNAs. Interestingly, the PPNAD microRNA signature reveals an aggressive phenotype that is similar to the microRNA profiles in malignant tissues. Specifically, the microRNA profile in PPNAD is characterized by the significant down-regulation of microRNAs (33/44) in comparison to normal adrenal. According to previous reports, in carcinogenesis there is mainly suppression of miRNA expression (88). Some of the microRNAs which are down-regulated in PPNAD such as let-7, miR-200b, miR-200c have tumor suppressor functions while others which were found upregulated; miR106b, miR-210 are related to increased cell growth and proliferation and regulate important signaling pathways as the MAPK and RAS pathways.

Let-7 family is a very important microRNA family in cancer. In most types of cancer or pre-cancerous conditions let-7 members are found down-regulated. Let-7 seems to

have a tumor suppressor role and its loss has been correlated with increased cell proliferation, activation of survival pathways and anti-apoptotic mechanisms (89, 90). In this study we detected 4 members of let-7 family (let-7a, let-7b, let-7c, let-7g) to be down-regulated in PPNADs. This result suggests that the aggressiveness and increased cell proliferation of PPNADs may be due to let-7 down-regulation. MiR-7 is down-regulated in glioblastomas and regulates EGFR and AKT pathways (91). MiR-132 and miR-146a are implicated in innate immunity responses and their down-regulation is followed by inhibition of interleukin signaling pathways. MiR-125b is frequently down-regulated in medulloblastomas and is involved in the Hedgehog (Hh) signalling pathway (92). MiR-135 is linked to colon carcinogenesis as well; it is suggested that it targets the adenomatous polyposis coli *(APC)* gene and affects the Wnt pathway (93). MiR-137 down-regulation accelerates glioblastoma formation (94). MiR-15b and miR-16 regulate the expression of the anti-apoptotic protein Bcl2 (95) MiR-335 is down-regulated in metastasis, acts as a tumor suppressor and regulates the expression of tenascin C *(TNC)* (96).

As far as it concerns the overexpressed microRNAs in both adrenal hyperplasias, MiR-106b is frequently over-expressed in cancer and promotes cell cycle progression through regulation of p21 (97) and E2F1 (98). MiR- 424 expression is modulated by the transcription factor PU.1 that controls monocyte-to-macrophage differentiation and excerts its effects by down-regulating the transcriptional factor Nuclear Factor I *(NFIA)* (99). Mir-210 is up-regulated in breast and lymphoid malignancies; it is regulated by the hypoxia inducible factor (*HIF*) (100).

Likewise, over-expressed microRNAs in MMAD/AIMAH (21/37) included several that have been implicated in tumorigenesis in other studies. MiR-196b is found up-regulated in leukemia and its expression pattern is associated to specific leukemia subtypes (101). Additionally, miR-196b expression is correlated with estrogen treatment (17beta-estradiol) in a new estrogen-mediated pathway via its target gene Hoxb8a in zebrafish (102). MiR-324-5p is implicated in medulloblastoma formation by deregulation of the Hedgehog (Hh) circuitry, specifically through targeting of the transcription factor *Gli1* (92). We found 11 microRNAs with increased expression in PPNAD such as miR-301, miR-139 and miR-594.

The biological significance of microRNAs is determined by the identification of their presumed gene targets. The available software-based algorithms predict 20-50 gene targets of each microRNA without taking into consideration the biological relevance of their interaction. For this reason it is important to match microRNA with expression data. Regarding the MMADs, we were able to match 20 microRNA-target gene pairs using our previously published expression data (17) (Table 3A). Examples of these pairs were the miR-210 and *HIF1A* and the miR-196a and *CPE*. MiR-210 expression has been found up-regulated in several cancer types and is associated with hypoxia- related pathways. Specifically, miR-210 is induced by *HIF1A* and hypoxic conditions; *HIF1A* binds in the promoter of miR-210 and induces its expression. The central role of HIF in the upregulation of miR-210 has been confirmed with studies in several types of cancer. In our previous study (17) *HIF1A* was found over-expressed (3.68-fold); our new finding, that miR-210 is also over-expressed in MMAD/AIMAH tissues, is consistent with the cDNA expression and other data such as fumarate hydratase (*FH*) mutations and adrenocortical tumors (103, 104) and the hypothesis that activation of the HIF1A pathway may be involved in MMAD/AIMAH pathogenesis (103). Studies in a renal carcinoma cell line showed that inactivating mutations in the VHL gene had as a result the suppression of HIF degradation and the induction of hypoxia and miR-210 upregulation (100). Hypoxia is a common feature in tumors with poor prognosis and insufficient response to drugs and miR-210 has been suggested as a prognostic factor for breast cancer (105).

CPE is found in neuroendocrine and endocrine cells such as pituitary and pancreatic β-cells, as a soluble exopeptidase as well as a receptor at the trans-Golgi network membrane; CPE converts prohormones into active neuropeptides and peptide hormones such as insulin, enkephalin and others and the CPE knockout mice have an endocrine phenotype (106). MiR-196a, which according to our findings regulates the expression of CPE, is reported so far as up-regulated in several cancers and has an oncogenic role in the regulation of Annexin A1 *(ANXA1)* in oesophageal cancer (107) and CPE has been found as a marker in neuroendocrine cancers (108).

We also identified two microRNAs, miR-130a and Mir-382 with an expression pattern that correlated with the diurnal cortisol levels of our MMAD patients, an index of the severity of their disease (Figure 3.10). MiR-130a has been found to be involved in cancer progression by regulating angiogenesis in endothelial cells through the suppression of two antiangiogenic homebox genes, *GAX* and *HOXA5* (109). MiR-382 has been studied in acute myeloid leukemia where a positive correlation between the leukemic subtype t(15;17)/PML-PARA and over-expression of miR-382 was detected (110). We also studied the correlation of the diurnal midnight cortisol levels and the microRNA profile in PPNAD. Let-7b was found negatively correlated to the above clinical marker, showing a potential role as a diagnostic marker for PPNAD.

Besides cAMP pathway, also Wnt signaling pathway has been associated with adrenal pathogenesis. Wnt signaling pathway regulates a vast range of cellular functions such as growth and differentiation and is critical during embryonic development (39). In Familial Adenomatous Polyposis (FAP), mutations of the adenomatosis polyposis coli (*APC*) gene activate the Wnt pathway and lead to the formation of adrenal adenomas (38), (4). Interestingly, expression studies in both MMAD/AIMAH (17) and PPNAD (10) have indicated overexpression of genes involved in the Wnt pathway such as axin1 (*AXIN1*), WNT1-inducible signaling pathway protein 2 (*WISP2*), catenin- β1 (*CTNNB1*) and glycogen synthase kinase -3β (*GSK3B*). Our findings here, suggest that when the PKA system is hyperactivated, miR-449 expression is down-regulated and WISP2 expression is increased.

We conclude that MMAD/AIMAH as well as PPNAD have a specific micro-RNA signature as other proliferative disorders. We suggest that microRNAs exhibit an important role in adrenal tumorigenesis and we describe a mechanism that might be involved in the early stages of adrenal tumorigenesis according to which the PKA system regulates the expression of miR-449 and induces the over-expression of WISP2 and the Wnt pathway in adrenal cortex.

Almost all types of benign lesions of the adrenal gland that were tested in this study exhibited functional abnormalities of the cAMP signaling pathway. These data indicate that more genetic defects of regulatory molecules of this pathway exist and

remain to be identified; they also suggest that pharmacological modulation of the cAMP signaling pathway should potentially be explored in the treatment of CS caused by benign ADTs.

Studies, like the present, will identify more pathways involved in adrenal pathogenesis and, as the clinical correlation above indicated, may even identify new biological markers of disease activity and or therapeutic targets in patients with ACTH-independent CS caused by BAHs.

5 References

References

1. Arthur C. Guyton, Human Physiology and Mechanisms of Disease, 1992
2. JE Skandalakis. Surgical Anatomy: The Embryologic And Anatomic Basis Of Modern Surgery 2004.
3. Stratakis, C. A. 2007. "Adrenocortical tumors, primary pigmented adrenocortical disease (PPNAD)/Carney complex, and other bilateral hyperplasias: the NIH studies." Horm Metab Res 39(6): 467-73
4. Bertherat J, Groussin L, Bertagna X. Mechanisms of disease: adrenocortical tumors--molecular advances and clinical perspectives. Nat Clin Pract Endocrinol Metab. 2006 Nov;2(11):632-41
5. Stratakis CA. Cushing syndrome caused by adrenocortical tumors and hyperplasias (corticotropin- independent Cushing syndrome. Endocr Dev. 2008;13:117-32
6. Stratakis CA, Boikos SA. Genetics of adrenal tumors associated with Cushing's syndrome: a new classification for bilateral adrenocortical hyperplasias. Nat Clin Pract Endocrinol Metab. 2007 Nov;3(11):748-57
7. Carney JA, Gordon H, Carpenter PC, Shenoy BV, Go VL.The complex of myxomas, spotty pigmentation, and endocrine overactivity. Medicine (Baltimore). 1985 Jul;64(4):270-83.
8. Kirschner LS, Carney JA, Pack SD, Taymans SE, Giatzakis C, Cho YS, Cho-Chung YS, Stratakis CA .Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex.Nat Genet. 2000 Sep;26(1):89-92
9. Boikos SA, Stratakis CA .Carney complex: pathology and molecular genetics. Neuroendocrinology.2006;83(3-4):189-99.
10. Horvath A, Mathyakina L, Vong Q, Baxendale V, Pang AL, Chan WY, Stratakis CA. Serial analysis of gene expression in adrenocortical hyperplasia caused by a germline PRKAR1A mutation. J Clin Endocrinol Metab. 2006 Feb;91(2):584-96.
11. Horvath A, Stratakis CA. Unraveling the molecular basis of micronodular adrenal hyperplasia. Curr Opin Endocrinol Diabetes Obes. 2008 Jun;15(3):227-33

12. Horvath A, Boikos S, Giatzakis C, Robinson-White A, Groussin L, Griffin KJ, Stein E, Levine E, Delimpasi G, Hsiao HP, Keil M, Heyerdahl S, Matyakhina L, Libè R, Fratticci A, Kirschner LS, Cramer K, Gaillard RC, Bertagna X, Carney JA, Bertherat J, Bossis I, Stratakis CA. A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. Nat Genet. 2006 Jul;38(7):794-800. Epub 2006 Jun 11
13. Bourdeau I, Stratakis CA 2002 Cyclic AMP-dependent signaling aberrations in macronodular adrenal disease. Ann N Y Acad Sci. Jun;968:240-55
14. Lacroix A, Ndiaye N, Tremblay J, Hamet P. 2001 Ectopic and abnormal hormone receptors in adrenal Cushing's syndrome. Endocr Rev. Feb;22(1):75-110
15. Kirk JM, Brain CE, Carson DJ, Hyde JC, Grant DB. 1999 Cushing's syndrome caused by nodular adrenal hyperplasia in children with McCune-Albright syndrome. J Petiatr Jun;134(6):789-92.
16. Fragoso MC, Domenice S, Latronico AC, Martin RM, Pereira MA, Zerbini MC, Lucon AM,Mendonca BB 2003 Cushing's syndrome secondary to adrenocorticotropin-independent macronodular adrenocortical hyperplasia due to activating mutations of GNAS1 gene. J Clin Endocrinol Metab. May;88(5):2147-51
17. Bourdeau I, Antonini SR, Lacroix A, Kirschner LS, Matyakhina L, Lorang D, Libutti SK, Stratakis CA 2004 Gene array analysis of macronodular adrenal hyperplasia confirms clinical heterogeneity and identifies several candidate genes as molecular mediators. Oncogene. Feb 26;23(8):1575-85
18. Soon PS, McDonald KL, Robinson BG, Sidhu SB. Molecular markers and the pathogenesis of adrenocortical cancer. Oncologist. 2008 May;13(5):548-61.
19. Aubert S, Wacrenier A, Leroy X, Devos P, Carnaille B, Proye C, Wemeau JL, Lecomte-Houcke M, Leteurtre E. Weiss system revisited: a clinicopathologic and immunohistochemical study of 49 adrenocortical tumors. Am J Surg Pathol. 2002 Dec;26(12):1612-9.
20. Icard P, Goudet P, Charpenay C, Andreassian B, Carnaille B, Chapuis Y, Cougard P, Henry JF, Proye C. Adrenocortical carcinomas: surgical trends and results of a 253-patient series from the French Association of Endocrine Surgeons study group. World J Surg. 2001 Jul;25(7):891-7.

21. Ringel MD, Schwindinger WF, Levine MA Clinical implications of genetic defects in G proteins. The molecular basis of McCune-Albright syndrome and Albright hereditary osteodystrophy. Medicine (Baltimore). 1996 Jul;75(4):171-84.
22. Lumbroso S, Paris F, Sultan C; European Collaborative Study.Activating Gsalpha mutations: analysis of 113 patients with signs of McCune-Albright syndrome--a European Collaborative Study. J Clin Endocrinol Metab. 2004 May; 89 (5):2107-13
23. Fragoso MC, Domenice S, Latronico AC, Martin RM, Pereira MA, Zerbini MC, Lucon AM, Mendonca BB. Cushing's syndrome secondary to adrenocorticotropin-independent macronodular adrenocortical hyperplasia due to activating mutations of GNAS1 gene J Clin Endocrinol Metab. 2003 May;88(5):2147-51.
24. Tasken et al. Signal Transduction in Health and disease, Advances in Second messenger and phosphoprotein research. Structure, function and regulation of Human cAMP-Dependent Protein Kinases. Vol 31,1997.
25. Stratakis CA, Kirschner LS, Carney JA. . Clinical and molecular features of the Carney complex: diagnostic criteria and recommendations for patient evaluation. J Clin Endocrinol Metab. 2001 Sep;86(9):4041-6
26. Horvath A, Bossis I, Giatzakis C, Levine E, Weinberg F, Meoli E, Robinson-White A, Siegel J, Soni P, Groussin L, Matyakhina L, Verma S, Remmers E, Nesterova M, Carney JA, Bertherat J, Stratakis CA Large deletions of the PRKAR1A gene in Carney complex. Clin Cancer Res. 2008 Jan 15;14(2):388-95
27. Greene EL, Horvath AD, Nesterova M, Giatzakis C, Bossis I, Stratakis CA. In vitro functional studies of naturally occurring pathogenic PRKAR1A mutations that are not subject to nonsense mRNA decay. Hum Mutat. 2008 May;29(5):633-9
28. Conti M, Beavo J Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem. 2007;76:481-511.
29. Zhang KY, Card GL, Suzuki Y, Artis DR, Fong D, Gillette S, Hsieh D, Neiman J, West BL, Zhang C, Milburn MV, Kim SH, Schlessinger J, Bollag G. A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. Mol Cell. 2004 Jul 23;15(2):279-86.

30. Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman J, Beavo JA, Phillips SC. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. Proc Natl Acad Sci U S A. 2000 Mar 28;97(7):3702-7
31. D'Andrea MR, Qiu Y, Haynes-Johnson D, Bhattacharjee S, Kraft P, Lundeen S. Expression of PDE11A in normal and malignant human tissues. J Histochem Cytochem. 2005 Jul;53(7):895-903
32. Loughney K, Taylor J, Florio VA. 3',5'-cyclic nucleotide phosphodiesterase 11A: localization in human tissuesInt J Impot Res. 2005 Jul-Aug;17(4):320-5
33. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev. 2006 Sep;58(3):488-520.
34. Horvath A, Giatzakis C, Robinson-White A, Boikos S, Levine E, Griffin K, Stein E, Kamvissi V, Soni P, Bossis I, de Herder W, Carney JA, Bertherat J,Gregersen PK, Remmers EF, Stratakis CA. Adrenal hyperplasia and adenomas are associated with inhibition of phosphodiesterase 11A in carriers of PDE11A sequence variants that are frequent in the population. Cancer Res. 2006 Dec 15;66(24):11571-5
35. Libé R, Fratticci A, Coste J, Tissier F, Horvath A, Ragazzon B, Rene-Corail F, Groussin L, Bertagna X, Raffin-Sanson ML, Stratakis CA, Bertherat J. Phosphodiesterase 11A (PDE11A) and genetic predisposition to adrenocortical tumors. Clin Cancer Res. 2008 Jun 15;14(12):4016-24. .
36. Boikos SA, Horvath A, Heyerdahl S, Stein E, Robinson-White A, Bossis I, Bertherat J, Carney JA, Stratakis CA. Phosphodiesterase 11A expression in the adrenal cortex, primary pigmented nodular adrenocortical disease, and other corticotropin-independent lesions. Horm Metab Res. 2008 May;40(5):347-53.
37. Horvath A, Mericq V, Stratakis CA. Mutation in PDE8B, a cyclic AMP-specific phosphodiesterase in adrenal hyperplasia. N Engl J Med. 2008 Feb 14;358(7):750-2.
38. Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest. 2002 Apr;109(8):987-91
39. Naylor EW, Gardner EJ. Adrenal adenomas in a patient with Gardner's syndrome. Clin Genet. 1981 Jul;20(1):67-73
40. Tissier F, Cavard C, Groussin L et al. Mutations of beta-catenin in adrenocortical tumors: activation of the Wnt signaling pathway is a frequent event in both

benign and malignant adrenocortical tumors. Cancer Res. 2005 Sep 1;65(17):7622-7

41. Gaujoux S, Tissier F, Groussin L Wnt/beta-catenin and 3',5'-cyclic adenosine 5'-monophosphate/protein kinase A signaling pathways alterations and somatic beta-catenin gene mutations in the progression of adrenocortical tumors. J Clin Endocrinol Metab. 2008 Oct;93(10):4135-40. Epub 2008 Jul 2
42. Esquela-Kerscher A, Slack FJ 2006 Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. Apr; 6(4):259-69
43. Filipowicz W, Bhattacharyya SN, Sonenberg N. 2008 Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. Feb;9(2):102-14
44. Kent OA, Mendell JT. 2006 A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene. Oct 9;25(46):6188-96
45. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. 2005 A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia N Engl J Med.Oct 27;353(17):1793-801.
46. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. 2007 MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA. May 2;297(17):1901-8
47. Lowery AJ, Miller N, McNeill RE, Kerin MJ. MicroRNAs as prognostic indicators and therapeutic targets: potential effect on breast cancer management Clin Cancer Res. 2008 Jan 15;14(2):360-5
48. Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, Wallace TA, Liu CG, Volinia S, Calin GA, Yfantis HG, Stephens RM, Croce CM. 2008 Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer Cancer Res. Aug 1;68(15):6162-70
49. Hu Z, Chen J, Tian T, Zhou X, Gu H, Xu L, Zeng Y, Miao R, Jin G, Ma H, Chen Y, Shen H 2008 Genetic variants of miRNA sequences and non-small cell lung cancer survival J Clin Invest. Jul;118(7):2600-8

50. Lin He and Gregory J.Hannon MicroRNAs: Small RNAs with a big role in gene regulation JULY 2004 | Vol 5, Nature Reviews.
51. Bourdeau I, Lampron A, Costa MH, Tadjine M, Lacroix A. 2007 Adrenocorticotropic hormone-independent Cushing's syndrome. Curr Opin Endocrinol Diabetes Obes, 14(3):219-25.
52. Boston BA, Mandel S, LaFranchi S, Bliziotes M. Activating mutation in the stimulatory guanine nucleotide-binding protein in an infant with Cushing's syndrome and nodular adrenal hyperplasia. J Clin Endocrinol Metab. 1994 Sep;79(3):890-3.
53. Mircescu H, Jilwan J, N'Diaye N, Bourdeau I, Tremblay J, Hamet P, Lacroix A. 2000 Are ectopic or abnormal membrane hormone receptors frequently present in adrenal Cushing's syndrome? J Clin Endocrinol Metab, 85(10):3531-6.
54. Bertherat J, Contesse V, Louiset E, Barrande G, Duparc C, Groussin L, Emy P, Bertagna X, Kuhn JM, Vaudry H, Lefebvre H. 2005 In vivo and in vitro screening for illegitimate receptors in adrenocorticotropin-independent macronodular adrenal hyperplasia causing Cushing's syndrome: identification of two cases of gonadotropin/gastric inhibitory polypeptide-dependent hypercortisolism. J Clin Endocrinol Metab, 90(3):1302-10.
55. Groussin L, Jullian E, Perlemoine K, Louvel A, Leheup B, Luton JP, Bertagna X, Bertherat J. 2002 Mutations of the *PRKAR1A* gene in Cushing's syndrome due to sporadic primary pigmented nodular adrenocortical disease. J Clin Endocrinol Metab, 87(9):4324-9.
56. Groussin L, Kirschner LS, Vincent-Dejean C, Perlemoine K, Jullian E, Delemer B, Zacharieva S, Pignatelli D, Carney JA, Luton JP, Bertagna X, Stratakis CA, Bertherat J.2002 Molecular analysis of the cyclic AMP-dependent protein kinase A (PKA) regulatory subunit 1A (*PRKAR1A*) gene in patients with Carney complex and primary pigmented nodular adrenocortical disease (PPNAD) reveals novel mutations and clues for pathophysiology: augmented PKA signaling is associated with adrenal tumorigenesis in PPNAD. Am J Hum Genet, 71(6):1433-42.
57. Groussin L, Horvath A, Jullian E, Boikos S, Rene-Corail F, Lefebvre H, Cephise-Velayoudom FL, Vantyghem MC, Chanson P, Conte-Devolx B, Lucas M, Gentil A, Malchoff CD, Tissier F, Carney JA, Bertagna X, Stratakis CA, Bertherat J. 2006 A *PRKAR1A* mutation associated with primary pigmented

nodular adrenocortical disease in 12 kindreds. J Clin Endocrinol Metab, 91(5):1943-9.

58. Bertherat J, Groussin L, Sandrini F, Matyakhina L, Bei T, Stergiopoulos S, Papageorgiou T, Bourdeau I, Kirschner LS, Vincent-Dejean C, Perlemoine K, Gicquel C, Bertagna X, Stratakis CA. 2003 Molecular and functional analysis of *PRKAR1A* and its locus (17q22-24) in sporadic adrenocortical tumors: 17q losses, somatic mutations, and protein kinase A expression and activity. Cancer Res, 63(17):5308-19.
59. Bourdeau I, Matyakhina L, Stergiopoulos SG, Sandrini F, Boikos S, Stratakis CA. 2006 17q22-24 chromosomal losses and alterations of protein kinase a subunit expression and activity in adrenocorticotropin-independent macronodular adrenal hyperplasia. J Clin Endocrinol Metab, 91(9):3626-32.
60. Gilman AG. A protein binding assay for adenosine 3':5'-cyclic monophosphate. Proc Natl Acad Sci U S A. 1970 Sep;67(1):305-12
61. Nesterova MV, Sashchenko LP, Vasiliev VY, Severin ES. A cyclic adenosine 3',5'-monophosphate-dependent histone kinase from pig brain. Purification and some properties of the enzyme.Biochim Biophys Acta. 1975 Feb 19;377(2):271-81.
62. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, Gross C, Engelhardt S, Ertl G, Bauersachs J. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. 2007 Circulation. Jul 17;116(3):258-67. Epub 2007 Jul 2. Circulation. Jul 17;116(3):e135.
63. Poletto R, Siegford JM, Steibel JP, Coussens PM, Zanella AJ. 2006 Investigation of changes in global gene expression in the frontal cortex of early-weaned and socially isolated piglets using microarray and quantitative real-time RT-PCR Brain Res. Jan 12;1068(1):7-15
64. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. 2008 The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1 Nat Cell Biol. May;10(5):593-601
65. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. 2007 MicroRNA signatures in human ovarian cancer Cancer Res. Sep 15;67(18):8699-707

66. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J. 2008 Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer Cancer Res. Apr 1;68(7):2094-105
67. Stratakis CA, Jenkins RB, Pras E, Mitsiadis CS et al. Cytogenetic and microsatellite alterations in tumors from patients with the syndrome of myxomas, spotty skin pigmentation, and endocrine overactivity (Carney complex). J Clin Endocrinol Metab. 1996 Oct;81(10):3607-14.
68. 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 Feb;93(2):565-71
69. Suwa T, Chen M, Hawks CL, Hornsby PJ. Zonal expression of dickkopf-3 and components of the Wnt signalling pathways in the human adrenal cortex. J Endocrinol. 2003 Jul;178(1):149-58
70. Katoh Y, Katoh M. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review) Int J Mol Med. 2008 Sep;22(3):271-5.
71. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. MicroRNA signatures in human ovarian cancer Cancer Res. 2007 Sep 15;67(18):8699-707.
72. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J. 2008 Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer Cancer Res. Apr 1;68(7):2094-105
73. Bueno MJ, Pérez de Castro I, Gómez de Cedrón M, Santos J, Calin GA, Cigudosa JC, Croce CM, Fernández-Piqueras J, Malumbres M 2008 Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression Cancer Cell. Jun;13(6):496-506
74. Lena AM, Shalom-Feuerstein R, di Val Cervo PR, Aberdam D, Knight RA, Melino G, Candi E. 2008 miR-203 represses 'stemness' by repressing DeltaNp63 Cell Death Differ. Jul;15(7):1187-95
75. Taganov KD, Boldin MP, Chang KJ, Baltimore D. 2006 NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12481-6.

76. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, Cheng JQ 2008 MicroRNA-155 Is Regulated by TGF{beta}/Smad Pathway and Contributes to Epithelial Cell Plasticity by Targeting RhoA Mol Cell Biol. Sep 15
77. Bandrés E, Cubedo E, Agirre X, Malumbres R, Zárate R, Ramirez N, Abajo A, Navarro A, Moreno I, Monzó M, García-Foncillas J. 2006 Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues Mol Cancer. Jul 19;5:29.
78. Nasser MW, Datta J, Nuovo G, Kutay H, Motiwala T, Majumder S, Wang B, Suster S, Jacob ST, Ghoshal K. 2008 Downregulation of microRNA-1 (miR-1) in lung cancer: Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin induced apoptosis bymiR-1J Biol Chem. Sep 25
79. Datta J, Kutay H, Nasser MW, Nuovo GJ, Wang B, Majumder S, Liu CG, Volinia S, Croce CM, Schmittgen TD, Ghoshal K, Jacob ST 2008 Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis Cancer Res. Jul 1;68(13):5049-58
80. El Ouaamari A, Baroukh N, Martens GA, Lebrun P, Pipeleers D, van Obberghen E 2008 miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells Diabetes.Oct;57(10):2708-17
81. Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda 2008 The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer. Nov;47(11):939-46
82. Chen YT, Kitabayashi N, Zhou XK, Fahey TJ 3rd, Scognamiglio T. 2008 MicroRNA analysis as a potential diagnostic tool for papillary thyroid carcinoma Mod Pathol. Sep;21(9):1139-46
83. Willenberg HS, Stratakis CA, Marx C, Ehrhart-Bornstein M, Chrousos GP, Bornstein SR. Aberrant interleukin-1 receptors in a cortisol-secreting adrenal adenoma causing Cushing's syndrome. N Engl J Med. 1998 Jul 2;339(1):27-31
84. Wong TS, Liu XB, Chung-Wai Ho A, Po-Wing Yuen A, Wai-Man Ng R, Ignace Wei W. 2008 Identification of pyruvate kinase type M2 as potential oncoprotein in squamous cell carcinoma of tongue through microRNA profilingInt J Cancer. Jul 15;123(2):251-7

85. Dore LC, Amigo JD, Dos Santos CO, Zhang Z, Gai X, Tobias JW, Yu D, Klein AM, Dorman C, Wu W, Hardison RC, Paw BH, Weiss MJ 2008 A GATA-1-regulated microRNA locus essential for erythropoiesis Proc Natl Acad Sci U S A. Mar 4;105(9):3333-8
86. Pase L, Layton JE, Kloosterman WP, Carradice D, Waterhouse PM, Lieschke GJ 2008 miR-451 regulates zebrafish erythroid maturation in vivo via its target gata2 Blood. Oct 10.
87. Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, Yang JM 2008 Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells Biochem Pharmacol. Sep 1;76(5):582-8
88. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, et al. MicroRNA expression profiles classify human cancers. Nature. 2005 Jun 9;435(7043):834-8
89. Stahlhut Espinosa CE, Slack FJ The role of microRNAs in cancer Yale J Biol Med. 2006 Dec;79(3-4):131-40.
90. Jay C, Nemunaitis J, Chen P, Fulgham P, Tong AW miRNA profiling for diagnosis and prognosis of human cancerDNA Cell Biol. 2007 May;26(5):293-300.
91. Kefas B, Godlewski J, Comeau L et al. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res. 2008 May 15;68(10):3566-72.
92. Ferretti E, De Smaele E, Miele E et al. Concerted microRNA control of Hedgehog signalling in cerebellar neuronal progenitor and tumour cells. EMBO J 2008 Oct 8;27(19):2616-27.
93. Nagel R, le Sage C, Diosdado B, et al. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. Cancer Res. 2008 Jul 15;68(14):5795-802.
94. Silber J, Lim DA, Petritsch C et al. miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med. 2008 Jun 24;6:14.
95. Xia L, Zhang D, Du R,et al. MiR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer. 2008 Jul 15;123(2):372-9.
96. Negrini M, Calin GA. Breast cancer metastasis: a microRNA story. Breast Cancer Res. 2008;10(2):203.

97. Ivanovska I, Ball AS, Diaz RL, Magnus JF, Kibukawa M, Schelter JM, Kobayashi SV, Lim L, Burchard J, Jackson AL, Linsley PS, Cleary MA 2008 MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression Mol Cell Biol. Apr;28(7):2167-74.

98. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, Iliopoulos D, Pilozzi E, Liu CG, Negrini M, Cavazzini L, Volinia S, Alder H, Ruco LP, Baldassarre G, Croce CM, Vecchione A 2008 E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer Cancer Cell. 2008 Mar;13(3):272-86

99. Rosa A, Ballarino M, Sorrentino A, Sthandier O, De Angelis FG, Marchioni M, Masella B, Guarini A, Fatica A, Peschle C, Bozzoni I 2007 The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiationProc Natl Acad Sci U S A. Dec 11;104(50):19849-54

100. Ivan M, Harris AL, Martelli F, Kulshreshtha R 2008 Hypoxia Response and microRNAs: No Longer Two Separate Worlds J Cell Mol Med. Jun 23

101. Schotte D, Chau JC, Sylvester G, Liu G, Chen C, van der Velden VH, Broekhuis MJ, Peters TC, Pieters R, Boer ML 2008 Identification of new microRNA genes and aberrant microRNA profiles in childhood acute lymphoblastic leukemia Leukemia. Oct 16

102. Cohen A, Shmoish M, Levi L, Cheruti U, Levavi-Sivan B, Lubzens E 2008 Alterations in micro-ribonucleic acid expression profiles reveal a novel pathway for estrogen regulation Endocrinology. Apr;149(4):1687-96

103. Matyakhina L, Freedman RJ, Bourdeau I, Wei MH, Stergiopoulos SG, Chidakel A, Walther M, Abu-Asab M, Tsokos M, Keil M, Toro J, Linehan WM, Stratakis CA. 2005 Hereditary leiomyomatosis associated with bilateral, massive, macronodular adrenocortical disease and atypical cushing syndrome: a clinical and molecular genetic investigation. J Clin Endocrinol Metab. Jun;90(6):3773-9.

104. Lehtonen HJ, Kiuru M, Ylisaukko-Oja SK, Salovaara R, Herva R, Koivisto PA, Vierimaa O, Aittomäki K, Pukkala E, Launonen V, Aaltonen LA. 2006 Increased risk of cancer in patients with fumarate hydratase germline mutation. J Med Genet. Jun;43(6):523-6.

105. Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, Harris AL, Gleadle JM, Ragoussis J 2008 hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer Clin Cancer Res. Mar 1;14(5):1340-8

106. Cawley NX, Zhou J, Hill JM, Abebe D, Romboz S, Yanik T, Rodriguiz RM, Wetsel WC, Loh YP 2004 carboxypeptidase E knockout mouse exhibits endocrinological and behavioral deficitsEndocrinology. Dec;145(12):5807-19

107. Luthra R, Singh RR, Luthra MG, Li YX, Hannah C, Romans AM, Barkoh BA, Chen SS, Ensor J, Maru DM, Broaddus RR, Rashid A, Albarracin CT 2008 MicroRNA-196a targets annexin A1: a microRNA-mediated mechanism of annexin A1 downregulation in cancers Oncogene. Jul 28

108. He P, Varticovski L, Bowman ED, Fukuoka J, Welsh JA, Miura K, Jen J, Gabrielson E, Brambilla E, Travis WD, Harris CC. 2004 Identification of carboxypeptidase E and gamma-glutamyl hydrolase as biomarkers for pulmonary neuroendocrine tumors by cDNA microarray. Hum Pathol. Oct;35(10):1196-209.

109. Chen Y, Gorski DH 2008 Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5 Blood. Feb 1;111(3):1217-26.

110. Li Z, Lu J, Sun M, Mi S, Zhang H, Luo RT, Chen P, Wang Y, Yan M, Qian Z, Neilly MB, Jin J, Zhang Y, Bohlander SK, Zhang DE, Larson RA, Le Beau MM, Thirman MJ, Golub TR, Rowley JD, Chen J 2008 Distinct microRNA expression profiles in acute myeloid leukemia with common translocationsProc Natl Acad Sci U S A. Oct 7;105(40):15535-40

Supplemental figures

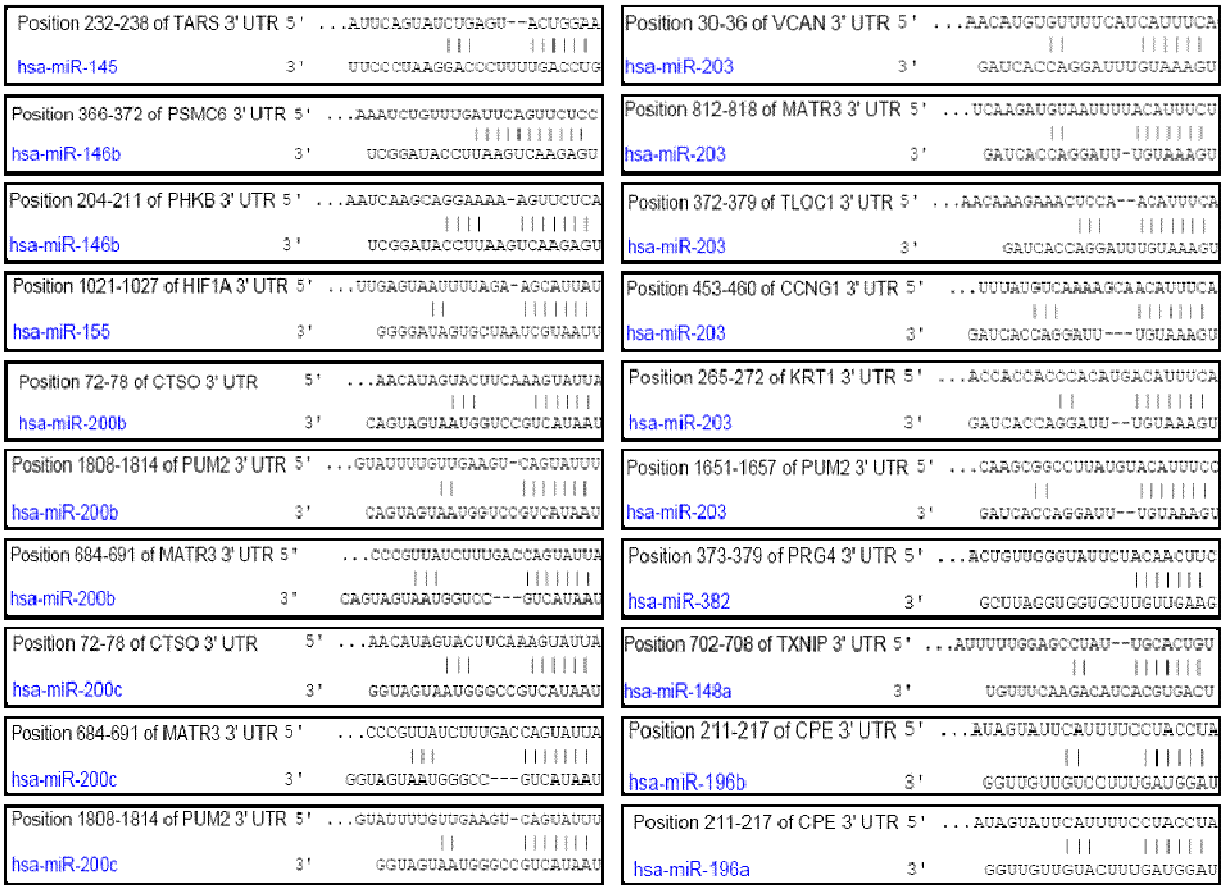


Figure 0.1: MicroRNA-target gene pairs sequences in MMAD according to TargetScan algorithm predictions.

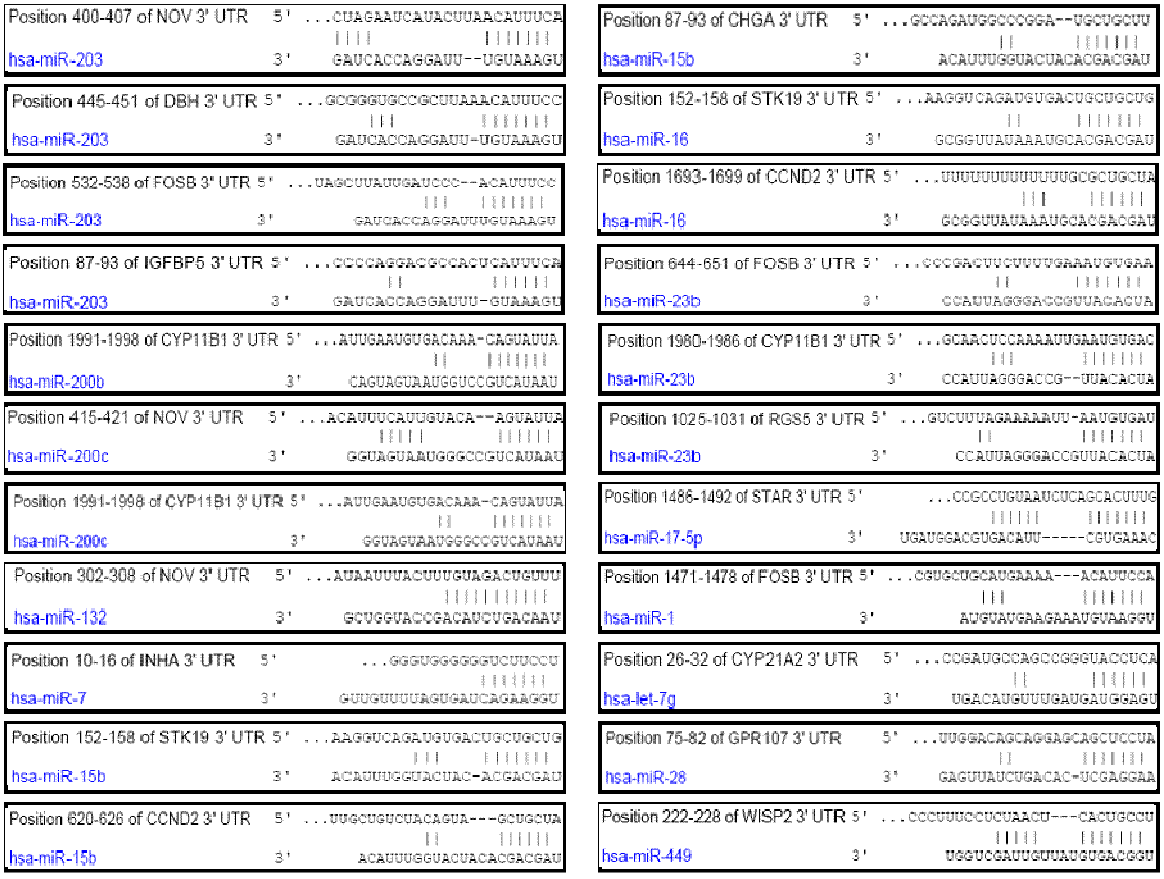


Figure 0.2: MicroRNA-target gene pairs sequences in PPNAD according to bioinformatic prediction algorithms.