Activation of Cyclic AMP Signaling Leads to Different Pathway Alterations in Lesions of the Adrenal Cortex Caused by Germline PRKAR1A Defects versus Those due to Somatic GNAS Mutations

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Context: The overwhelming majority of benign lesions of the adrenal cortex leading to Cushing syndrome are linked to one or another abnormality of the cAMP or protein kinase pathway. *PRKAR1A*-inactivating mutations are responsible for primary pigmented nodular adrenocortical disease, whereas somatic *GNAS* activating mutations cause macronodular disease in the context of McCune-Albright syndrome, ACTH-independent macronodular hyperplasia, and, rarely, cortisol-producing adenomas.

Objective and Design: The whole-genome expression profile (WGEP) of normal (pooled) adrenals, *PRKAR1A*- (3) and *GNAS*-mutant (3) was studied. Quantitative RT-PCR and Western blot were used to validate WGEP findings.

Results: MAPK and p53 signaling pathways were highly overexpressed in all lesions against normal tissue. *GNAS*-mutant tissues were significantly enriched for extracellular matrix receptor interaction and focal adhesion pathways when compared with *PRKAR1A*-mutant (fold enrichment 3.5, $P < 0.0001$ and 2.1, $P < 0.002$, respectively). *NFKB*, *NFKBIA*, and *TNFRSF1A* were higher in *GNAS*-mutant tumors ($P < 0.05$). Genes related to the *Wnt* signaling pathway (*CCND1*, *CTNNB1*, *LEF1*, *LRP5*, *WISP1*, and *WNT3*) were overexpressed in *PRKAR1A*-mutant lesions.

Conclusion: WGEP analysis revealed that not all cAMP activation is the same: adrenal lesions harboring *PRKAR1A* or *GNAS* mutations share the downstream activation of certain oncogenic signals (such as MAPK and some cell cycle genes) but differ substantially in their effects on others. (***J Clin Endocrinol Metab* 97: E687–E693, 2012**)

Abbreviations: AIMAH, ACTH-independent macronodular adrenal hyperplasia; CNC, Carney complex; CS, Cushing syndrome; DAVID, Database for Annotation, Visualization, and Integrated Discovery; ECM, extracellular matrix; IKBa, inhibitory-κB; MMAD, massive macronodular adrenocortical disease; NFKB, nuclear factor-κB; PDE, phosphodiesterase; PGE2, prostaglandin E2; PKA, protein kinase A; PPNAD, primary pigmented nodular adrenocortical disease; qRT-PCR, quantitative real-time PCR; RAB-13, renin-angiotensin system oncogene family 13; WGEP, whole-genome expression profile.

The majority of benign lesions of the adrenal cortex leading to Cushing syndrome are linked to one or another abnormality of the cAMP/protein kinase A (PKA) signaling pathway (1–3). Inactivating mutations of the *PRKAR1A* gene coding for the regulatory 1α-subunit of PKA are responsible for Carney complex (CNC) in the majority of patients (4). Primary pigmented nodular adrenocortical disease (PPNAD) is the most common endocrine tumor associated with CNC, leading to Cushing syndrome in more than 60% of the patients (4). Micronodular adrenocortical hyperplasias and some cortisol-producing adenomas are associated with phosphodiesterase (PDE) 11A and phosphodiesterase 8B defects, coded, respectively, by the *PDE11A* and *PDE8B* genes (5–8).

Somatic *GNAS* mutations have been described only in a small number of both ACTH-independent macronodular adrenal hyperplasia (AIMAH), also known as massive macronodular adrenocortical disease (MMAD), rarely in cortisol-producing adenomas (3, 9), and in the context of McCune-Albright syndrome. cAMP/PKA signaling is altered in MMAD as in adrenal tumors with 17q losses or *PRKAR1A* mutations (1, 10). Furthermore, common adrenal lesions without germline or somatic *GNAS*, *PRKAR1A*, *PDE11A*, and *PDE8B* mutations and associated with ACTH-independent Cushing syndrome (CS) had functional abnormalities of the cAMP signaling pathway, such as increased cAMP levels, decreased total PDE, and/or increased PKA activity (2). Thus, aberrations of cAMP/PKA signaling are essential in the pathogenesis of benign cortisol-producing lesions of the adrenal cortex.

Previous transcriptome studies demonstrated the overexpression of genes that regulate or are part of the *Wnt* signaling pathway in both PPNAD and MMAD without *GNAS* mutations (11, 12). In agreement with these findings, a whole-genome transcriptome profiling of tumors produced by mouse models of *Prkar1a* haploinsufficiency identified *Wnt* signaling as the main pathway activated by abnormal cAMP signaling along with cell cycle abnormalities (13).

In this study, we analyzed the whole-genome expression profile (WGEP) of PPNAD caused by germline *PRKAR1A* mutations and adrenal lesions harboring somatic *GNAS* mutations. The data indicated that cAMP activation in adrenal lesions harboring *PRKAR1A* or *GNAS* mutations share the downstream activation of some oncogenic signals but differ significantly in their effects on others. These findings are important for the design of molecularly targeted therapies for benign adrenal tumors or hyperplasias leading to CS.

Patients and Methods

Subjects, tissues, DNA studies, and sequencing

Patients were studied under protocols 95CH0059 and 00CH160, both approved by the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD). We studied two groups of lesions (Table 1): group 1 included three microdissected PPNAD samples from patients with CNC caused by three different nonsense *PRKAR1A* mutations (see text below); and group 2 included three microdissected samples from adrenal lesions that

TABLE 1. Clinical and laboratory data from patients with PPNAD and adrenal masses with *GNAS* mutation

Patient code	Adrenal disease	Age at diagnosis	UFC (8–88 μg per 24 h)	Midnight cortisol (<1.8 μg/dl)	After high dose Dex/ after Liddle's urinary costisol[a]	Imaging	Other tumors/conditions	Gene	Mutation
CAR47.01	PPNAD	31 yr	24.5	5.5	180.6 μg per 24 h	Suggestive of nodular disease	Left atrial myxoma/multiple ischemic strokes	*PRKAR1A*	c.177 + 1G>A
CAR21.02	PPNAD	20 yr	226.4	11.1	12.1 μg/dl (serum)	Two right adrenal masses (the largest 2 × 1.5 cm)/enlargement of left adrenal gland	Liver tumors (hepatocellular adenoma), pituitary GH- secreting microadenoma, depression	*PRKAR1A*	c.101_105delCTATT/ p.Ser34fsX9
CAR20.14	PPNAD	13.8 yr	25	5.8	110 μg per 24 h	Suggestive of nodular disease	Recurrent atrial myxomas	*PRKAR1A*	c.491_492delTG/ p.Val164fsX4
ADTLG01	AIMAH/MMAD	30 yr, recurrence at 36 yr	103	7.6	8.3 μg/dl	2 cm right adrenal tumor/2.2 cm left adrenal tumor	No	*GNAS*	R201H
CAR588.03	McCune-Albright syndrome	1 month	NA	NA	NA	Bilateral adrenal enlargement with numerous small nodules	Thyrotoxicosis, diabetes, café-aux-lait spots, fibrodysplastic lesions	*GNAS*	R201H

NA, Not available; UFC, urinary free cortisol; Dex, dexamethasone.

[a] High-dose Dex: overnight 8 mg dexamethasone test, Liddles's 6-d test.

were all caused by the same somatic *GNAS* mutation (p.R201H): a cortisol-producing adenoma from a patient with AIMAH/MMAD and CS and two microdissected adenomatous nodules from a patient with MAS and CS (Supplemental Material, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

All tissue samples were carefully dissected out of surrounding periadrenal fat, adrenal capsule, and other structures. In tissues with *GNAS* mutations, only cortical nodular tissue that was previously sequenced and found to contain the p.R201H mutation was used for the RNA studies. In the case of PPNAD, all samples contained the *PRKAR1A* mutation in the heterozygote state; special attention was paid to not include RNA from *PRKAR1A*-haploinsufficient nodules [those that have undergone loss of heterozygosity (LOH) for the 17q22-24 locus of *PRKAR1A*] because these tissues may have additional genetic and transcriptome changes. Sequencing for the *PRKAR1A* and *GNAS* genes was done as has been described elsewhere (9, 10). The three *PRKAR1A* mutations were c.177 + 1G>A, c.101_105delCTATT, and c.491_492delTG, respectively; they all lead to predicted truncated proteins and their mRNA undergo nonsense-mediated decay (NMD) and are not expressed (4).

Microarray analysis

Total RNA extraction was performed from all tissues using the Trizol reagent method (Invitrogen, Carlsbad, CA). Samples were further purified using the RNeasy columns (QIAGEN, Valencia, CA), and the quality of RNA was assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Three commercially available pools of human adrenal total RNA (CLONTECH, Mountain View, CA; BioChain, Hayward, CA; Ambion, Austin, TX) were used as reference samples. The normal reference RNA that was used was total adrenal-derived RNA. Preparation of cRNA from total RNA, hybridization in Sentrix HumanRef-8 Expression BeadChips (Illumina, San Diego, CA), scanning, and image analysis was done as previously described (14). Analysis of Illumina data were performed using Illumina BeadStudio software (Illumina), which returns the trimmed mean average intensity for each single gene probe type (nonnormalized). Any gene consistently with a *P* detection value above 0.01 for all samples was eliminated from further analysis. Z-transformation for normalization was performed for each Illumina sample/array (14). First, the raw intensity data for each sample were $\log_{10}$ transformed, and Z scores were calculated by subtracting the overall average gene intensity (within a single experiment) from the raw intensity data for each gene and then dividing that result by the SD of all of the measured intensities. Changes in gene expression (ratio) between different Z-transformed data sets (nodules compared with the average of the normal adrenal pools) were calculated as differences between the corresponding Z scores and then divided by the SD of each Z difference data set (14). A 2-fold change was used as the cutoff value to identify over- and underexpressed genes in adrenal nodules in relation to normal adrenal pools. Microarray data are in compliance with the Minimal Information About a Microarray Experiment format. The raw and normalized microarray data reported in this paper have been deposited in the Gene Expression Omnibus database (accession no. GSE 33694).

Analysis of mRNA profiling and validation

The WGEP was analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatic Resources 2008 (National Institute of Allergy and Infectious Diseases, National Institutes of Health, http://david.abcc.ncifcrf.gov/home.jsp) (15, 16). The lists of genes (induced or repressed) were submitted to the DAVID database genes are clustered according to a series of common key words. The proportion of each key word in the list is compared with the one in the whole genome, making it possible to compute *P* values and enrichment scores (geometric mean of the inverse log of each *P* value). The detailed information of gene alterations was systematically reported on Kyoto Encyclopedia of Genes and Genomes (KEEG) pathways.

Quantitative real-time PCR (qRT-PCR) was performed in the ABI Prism 7700 sequence detector using PCR array plates (SABiosciences, Frederick, MD) and TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The assay identifications were: *NFKB1*, Hs00765730_m₁; *RAB13*, Hs00762784_s₁; *RAPGEF4* (*EPAC2*), Hs00899815_m₁; *BMP4*, Hs00370078_m₁; and 18S rRNA, 4352930E. The average of three commercially available pools of human adrenal total RNA (CLONTECH, BioChain, and Ambion) was used as control. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method (17).

Protein studies

Western blot analysis was performed following standard procedures (13). The following primary antibodies were used: beta catenin (CTNNB1; 9587; Cell Signaling, Beverly, MA), nuclear factor-κB (NFKB; ab16502; Abcam, Cambridge, MA), inhibitory-κB (IKBa; ab32518; Abcam), renin-angiotensin system oncogene family 13 (RAB-13; ab55889; Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab9485; Abcam). All immunohistochemistry was performed at Histoserve, Inc. (Germantown, MD) using standard procedures. The following primary antibodies were used: CTNNB1 (9587; Cell Signaling), NFKB (ab16502; Abcam), and IKBa (ab32518; Abcam). The following grading system was used for staining evaluation: negative (absence of expression), weak staining (from 1 to 25% of immunoreactive cells), and strong staining (>25% of immunoreactive cells). We also measured the PKA enzymatic activity in tissue extracts as previously described (18).

Statistical analysis

All statistical analyses were performed with the SPSS 16.0 (SPSS Inc., Chicago, IL). Continuous data are expressed as mean ± SD. All the experiments were performed in triplicate. A two-sample *t* test was used for paired samples. We considered a *P* < 0.05 significant for all comparisons.

Results

Transcriptome analysis, validation, and PKA activity

The gene set enrichment analysis of the WGEP revealed that signaling for P53 and MAPK was significantly enriched in both *PRKAR1A*- and *GNAS*-mutant lesions. Adrenal masses with *GNAS* mutations were significantly en-

riched with extracellular matrix (ECM) receptor interaction and focal adhesion pathways when compared with PPNAD (fold enrichment 3.5, $P < 0.0001$, and 2.2, $P < 0.002$, respectively) (Fig. 1A). The overexpressed genes in *GNAS*-mutant lesions compared with those with the *PRKAR1A* mutations included *NFKB1* [nuclear factor of κ-light polypeptide gene enhancer in B cells 1; ratio ($\log_{10}$) 4.2, $P = 0.004$], *ELK1* (member of the ETS oncogene family; ratio 3.0, $P = 0.03$), *LPAR1* (lysophosphatidic acid receptor 1; ratio 12.6, $P = 0.04$), *RAF1* (v-raf-1 murine leukemia viral oncogene homolog 1; ratio 5.3, $P =$ 0.04), *TNFRSF1A* (tumor necrosis factor receptor superfamily, member 1A; ratio 4.8, $P = 0.05$). Expression data were then validated by quantitative PCR and/or Western blot. Indeed, *NFKB* and *NFKBIA* expression was higher in *GNAS*-mutant tumors than in PPNAD samples at messenger and protein levels ($P < 0.05$) (Fig. 1, B–D). *EPAC-2* and *RAB13*, which are activated by a cAMP-dependent but PKA-independent mechanism, were highly expressed in adrenal tumors with *GNAS* mutations at the mRNA and protein levels (Fig. 1, B and C) but not in those with *PRKAR1A* mutations.
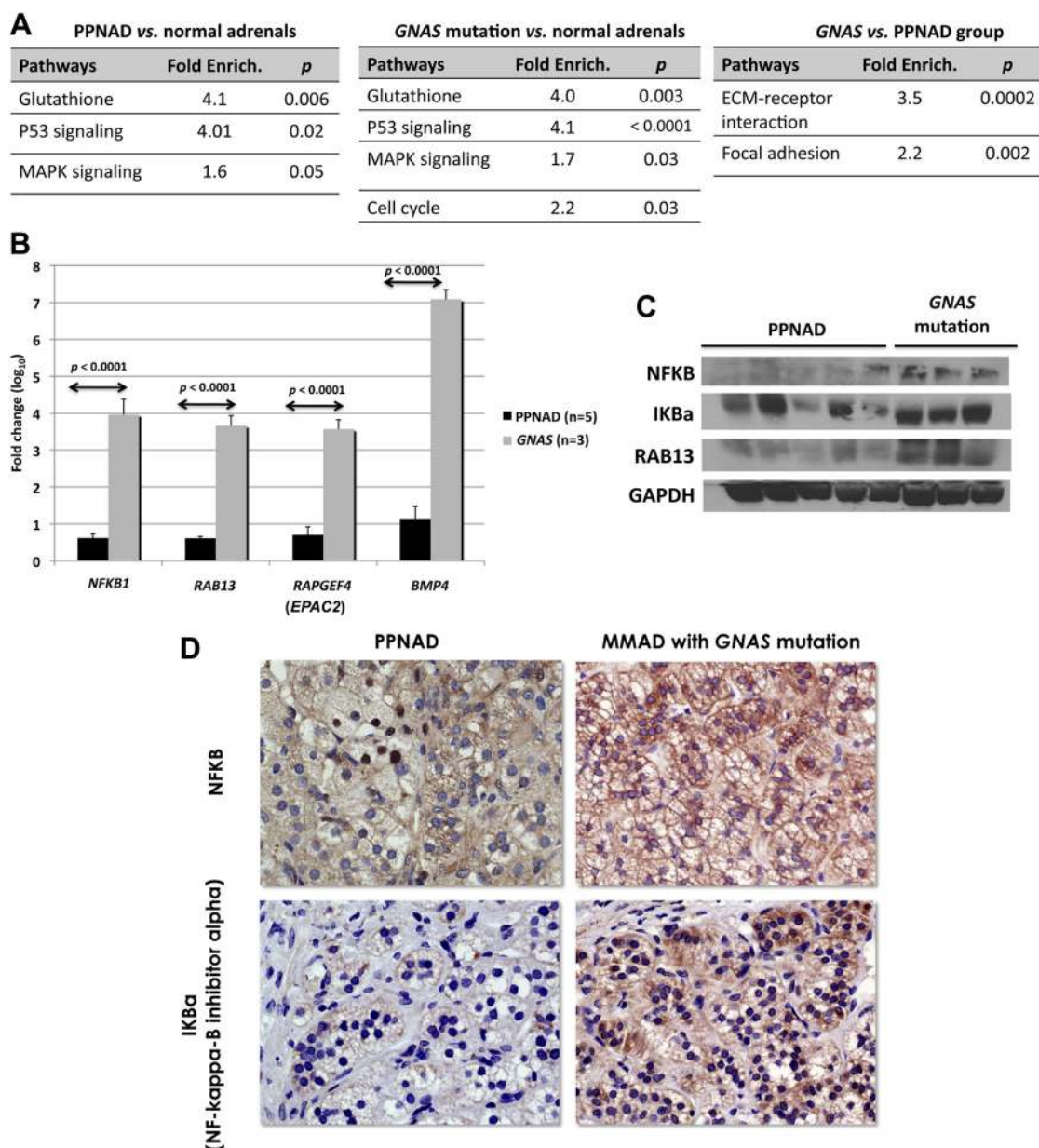

FIG. 1. A, Functional analysis of whole-genome transcriptome profiling of PPNAD and adrenal masses harboring the *GNAS* mutation. The array functional analysis was performed using DAVID Bioinformatics Resources 2008, National Institute of Allergy and Infectious Diseases, National Institutes of Health (http://david.abcc.ncifcrf.gov/home.jsp). B, Expression of *NFKB1, RAB13, EPAC2* and *BMP4* in the *GNAS* mutant lesions compared with PPNAD by quantitative PCR. C, NFKB1, IKBa, and RAB13 proteins by Western blot. D, NFKB1 and IKBa staining was stronger in AIMAH/MMAD with the R201H *GNAS* mutation than in a *PRKAR1A*-mutant PPNAD. IMAH, ACTH-independent macronodular adrenocortical hyperplasia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
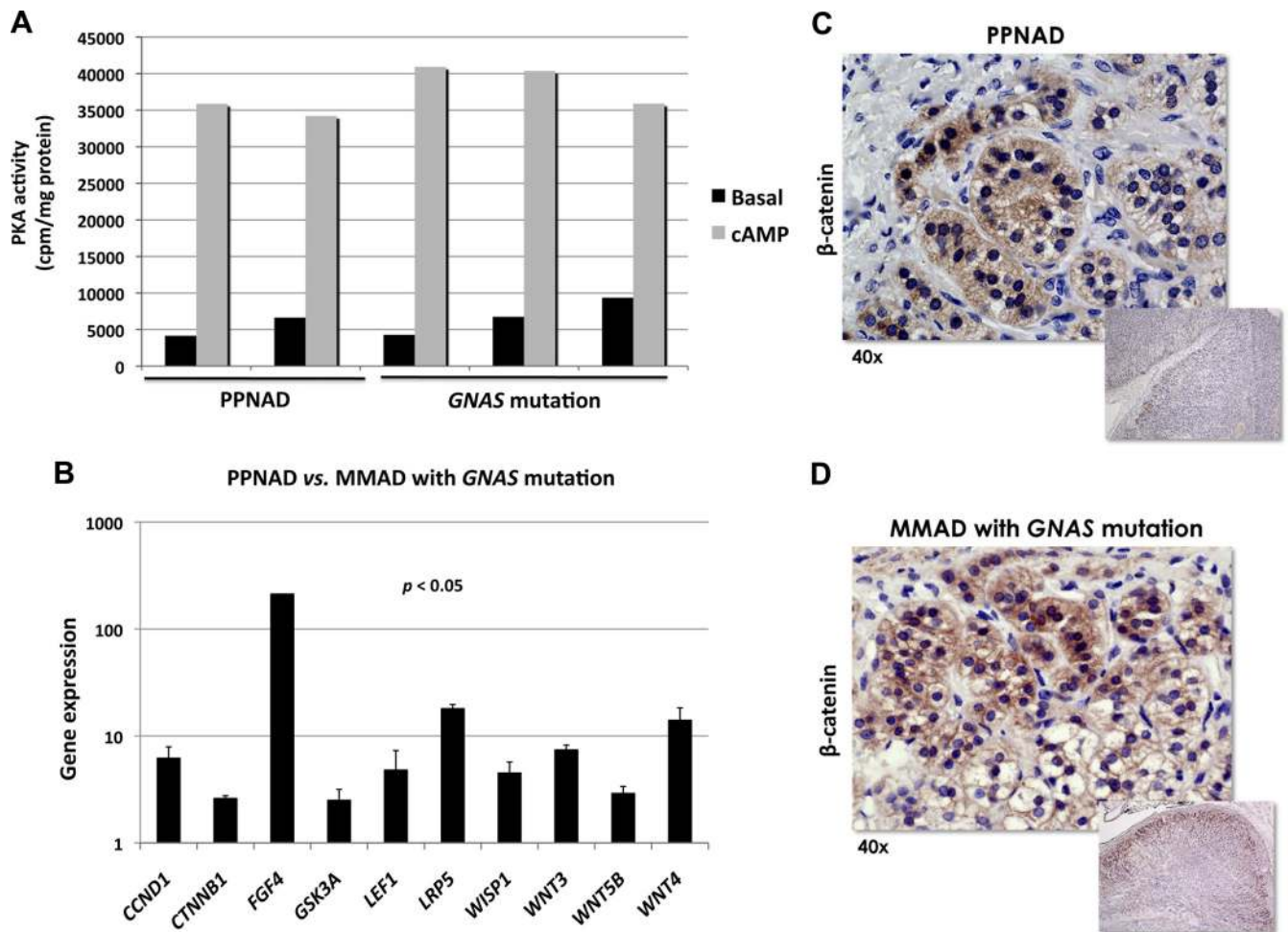
FIG. 2. A, Basal and cAMP-stimulated PKA activity in *PRKAR1A*- and *GNAS*-mutant lesions. B, A qRT-PCR array including 84 genes involved in *Wnt* signaling demonstrated that key oncogenes were overexpressed in *PRKAR1A*- *vs.* *GNAS*-mutant lesions. C and D, β-Catenin staining was strong in both *PRKAR1A*- and *GNAS*-mutant lesions, but interestingly only in nodules in the former.

We also analyzed the overlap between the *PRKAR1A*- and *GNAS*-mutant lesions. *FATE1*, *FOSB*, *CXCR4*, *NOV*, *PDE2A*, *NR4A1*, *NR5A1*, *NR0B1*, *FGF9*, and *CASP9* were among the genes overexpressed in both groups (Supplemental Table 1). On the other hand, *TH*, *CART*, *CHCB*, *RBP7*, *RGS4*, *IGFBP4*, and *IGFBP3* were among the genes underexpressed in both *PRKAR1A*- and *GNAS*-mutant lesions (Supplemental Table 2).

This variability in gene expression was not accompanied by significant differences in PKA activity: the latter was measured in five samples (Fig. 2A). After cAMP exposure, PKA activity was similar in PPNAD and *GNAS*-mutant tumors (35.046 ± 4.753 *vs.* 39.062 ± 2.757 cpm/mg protein).

Cancer pathways

A qRT-PCR array carrying 84 genes of the *Wnt* signaling pathway was tested in both *GNAS*- and *PRKAR1A*-mutant samples. *CCND1*, *CTNNB1*, *LEF1*, *LRP5*, *WISP1*, and *WNT3* were overexpressed in *PRKAR1A*-mutant lesions (Fig. 2B). Immunohistochemistry for

β-catenin *per se* was strongly positive (nuclear and cytoplasmic) in both the PPNAD and *GNAS*-mutant lesions (Fig. 2, C and D), but only in nodules in the former.

Apoptosis, cancer, and cell cycle pathways were also studied and compared with normal adrenal pools. Among 84 genes included in the apoptosis array plate, *IGF1R*, *CASP3*, *CARD6*, *BAG1*, and *ABL1* were significantly overexpressed in *PRKAR1A*-mutant samples (Supplemental Table 3). In addition, expression of the oncogenes *FGFR2* and *RAF1* was significantly higher in PPNAD (Supplemental Table 4). Cell cycle genes were also found to be overexpressed in PPNAD (Supplemental Table 5), including *CDK6*, *CDK8*, *CCNC*, and *CCNT1*.

Discussion

In this study, we characterized the expression signature of PPNAD caused by *PRKAR1A*-truncating mutations and adrenal masses harboring somatic *GNAS* mutations. In other words, we studied two adrenal lesions that are both

caused by genetic defects, leading to increased cAMP/PKA signaling. The MAPK and p53 signaling pathways were enriched in both the *PRKAR1A*- and *GNAS*-mutant lesions, but a significant enrichment with the ECM receptor interaction and focal adhesion pathways characterized only the latter. Indeed, proteins involved with ECM receptor interaction and adhesion, such as integrins and their ligands, are induced by EPAC2 (exchange protein directly activated by cAMP; cAMP-GEFII; RapGEF4) activation through a cAMP-dependent but PKA-independent mechanism (19). Our transcriptome analysis revealed that EPAC2 is overexpressed in MMAD with a *GNAS* mutation.

NFKB was overexpressed in *GNAS*-mutant lesions. Lee *et al.* (20) showed that activation of NFKB, MAPK, and p300 are essential for IL-1B-induced cytosolic phospholipase A2 expression and prostaglandin E2 (PGE2) secretion. We recently demonstrated that increased PKA led to inflammation-independent activation of caspase-1 via overexpression of the protooncogene (and early osteoblast factor) *Ets-1* (21). Caspase-1 activation promotes IL-1B production, which is a potent stimulator of cyclooxygenase-2 and microsomal prostaglandin E synthase-1, the main enzymes to PGE2 elevation. High PGE2 levels in cells with defective PKA regulation increased cAMP levels and in turn activated Wnt signaling, like in other states of inappropriate PKA activity (21).

The findings of this study have important implications for therapeutics because the two pathways (NFKB and PKA) can now be targeted interchangeably (or, maybe, simultaneously) in adrenal lesions associated with either *GNAS* mutation or PKA defects. Interestingly, IL-1B antagonists and caspase-1 inhibitors have been proposed as useful drugs to control the bone manifestations of patients with PKA defects or McCune-Albright syndrome (21).

This study shows the overexpression of key genes that are part of *Wnt* signaling and other cancer pathways in PPNAD; β-catenin expression was strong in both *PRKAR1A*- and *GNAS*-mutant lesions, although in the former only within the nodules. Previous expression studies in PPNAD and AIMAH/MMAD demonstrated the overexpression of genes involved in *Wnt* signaling activation (11, 12, 22). Somatic mutations of the *CTNNB1* gene have been found in adrenal tumors from patients with PPNAD and CNC caused by germline *PRKAR1A*-inactivating mutations (23, 24). Recently a microRNA profile analysis showed that cAMP and/or PKA via microRNA regulation affects the *Wnt* signaling pathway in both PPNAD (25) and AIMAH/MMAD (26). miR-449 was one of the highest down-regulated microRNA in PPNAD and is predicted to regulate *WISP2*, a gene that we had also identified as overexpressed in the adrenal glands of patients

with AIMAH/MMAD and food-dependent CS (12). The present study confirms that *Wnt* signaling is an important mediator of adrenal tumors related to *PRKAR1A* mutations, probably more so than those due to *GNAS* mutations.

In conclusion, this report demonstrates that not all increased cAMP/PKA signaling is the same with regard to adrenocortical tumor formation. Clearly the role of non-PKA-dependent functions of cAMP in the adrenal cortex has not been adequately investigated. These data also show that cAMP signaling inhibitors will have to be titrated for non-PKA-dependent effects if they are ever to be used as molecularly designed therapies for subclinical CS in the context of bilateral adrenal hyperplasias.

Acknowledgments

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