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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[1]

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ABSTRACT

Germ-line protein kinase A (PKA) regulatory-subunit type-Iα (RIα; *PRKAR1A*)-inactivating mutations and loss-of-heterozygosity (LOH) of its 17q22–24 locus have been found in Cushing syndrome (CS) caused by primary pigmented nodular adrenocortical disease (PPNAD). We examined whether somatic 17q22–24, *PRKAR1A*, or PKA changes are present in 44 sporadic adrenocortical tumors (29 adenomas and 15 cancers); 26 of these tumors were responsible for CS. A probe containing the *PRKAR1A* gene–mapped by fluorescent *in situ* hybridization to 17q22–24–and corresponding microsatellite markers were used to study allelic losses; *PRKAR1A* was sequenced in all samples. 17q22–24 losses were seen in 23 and 53% of adenomas and cancers, respectively. In three tumors, somatic, *PRKAR1A*-inactivating mutations were identified: (*a*) a nonsense mutation in exon 6 (A751G); (*b*) a splicing mutation (9IVS-1G/A); and (*c*) a transition (1050T>C) followed by a 22-bp deletion, also in exon 9; all predicted premature RIα protein terminations. Quantitative message and protein studies showed RIα down-regulation in tumors with genetic changes; their cortisol secretion pattern was similar to that of PPNAD, and they had higher PKA activity by enzymatic studies. We conclude that somatic allelic losses of the 17q22–24 region, *PRKAR1A*-inactivating mutations or down-regulation, and corresponding PKA activity changes are present in at least some sporadic adrenocortical tumors, especially those with a PPNAD-like clinical presentation of CS.

INTRODUCTION

Despite the relatively early discovery of the *TP53* gene, as one of the primary tumor suppressor genes involved in ACT[4] formation (1, 2), few other genetic defects that are associated with this process have been identified (3, 4). Factors that have been implicated in adrenal tumorigenesis include lack of 11p15 imprinting and overexpression of the insulin-like growth factor type 2 (*IGF2*) gene (5, 6), alterations of inhibin A (*INHA*), and/or its 2q locus (7, 8), yet unidentified genes on 2p16 (9) and 9q34 (10), and various substances or transcription factors, including urotensin II (11), *novH* (12), and the cAMP response-element binding protein and modulator (13, 14).

Various components of the cAMP-dependent PKA signaling pathway, including the ACTH-receptor (*MC2R* gene; Ref. 15) and the $G_s\alpha$ subunit (*GNAS1*; Ref. 16), have also been implicated in ACT pathogenesis (17, 18). Recently, the gene coding for the PKA regulatory-subunit type-I α (RIα), *PRKAR1A*, was found to be responsible for most cases of a relatively rare form of bilateral adrenocortical hyperplasia, PPNAD (19, 20). Germ-line *PRKAR1A*-inactivating mutations were found in both patients with isolated PPNAD and those with Carney complex (CNC), a multiple neoplasia syndrome with PPNAD as its main endocrine manifestation (21, 22). In PPNAD, as in other CNC tumors, LOH of the 17q *PRKAR1A* locus and an abnormal PKA activity have been demonstrated, suggesting that *PRKAR1A* acts, perhaps, as a tumor suppressor gene (22, 23), despite some *in vitro* evidence that RIα is overexpressed in cancer cell lines and may be a target for chemotherapy of certain tumors (24) or that PKA suppresses proliferation of leukemic T cells (25).

PPNAD may cause "classic" CS or an insidious, clinically atypical form of hypercortisolism, which may be associated with cyclical CS, simple disturbances of the normal circadian variation of cortisol secretion, and/or other atypical features (26). Most of these patients present with osteoporosis, and, occasionally, myopathy and cachexia, but tend to lack severe obesity, persistent hypertension, moon facies, and other manifestations of CS (26, 27). Patients with PPNAD respond to low and high doses of DXM with progressively increasing glucocorticoid excretion, a "paradoxical" response that may be used diagnostically to identify otherwise asymptomatic carriers or to distinguish PPNAD from other adrenocortical tumors (27, 28).

In this study, we examined whether somatic alterations of the *PRKAR1A* gene were associated with the development of sporadic ACTs in 44 patients, including 26 with ACTH-independent CS. Thus far, somatic *PRKAR1A* locus and gene changes have only been described in thyroid tumors (29). The limitations of LOH studies with polymorphic markers that are identified in the proximity of the gene of interest by sequence tag (STS)-mapping are well known. DNA from normal tissue is not always available, some markers are uninformative, and small deletions can be missed. Thus, in the present study, in addition to LOH, we used a BAC that contains the *PRKAR1A* gene to study allelic losses in several of the tumors. Our findings indicated that 17q22–24 allelic losses are frequent in ACTs. The *PRKAR1A* gene was sequenced in all samples; novel mutations were identified in three adenomas from unrelated patients, all with paradoxical responses to DXM and one with cyclical CS, suggesting a *PRKAR1A* genotype-clinical phenotype correlation, for the first time in sporadic ACTs. We also studied RIα expression and PKA activity in available samples and found that genetic alterations of PRKAR1A and/or its 17q22–24 locus correlated with decreased RIα expression and alterations in PKA activity.

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[4] The abbreviations used are: ACT, adrenocortical tumor; PKA, protein kinase A; LOH, loss of heterozygosity; PPNAD, primary pigmented nodular adrenocortical disease; DXM, dexamethasone; CS, Cushing syndrome; ACTH, adrenocorticorticotropic hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AC, adrenal cancer; CNC, Corney complex; PKI, protein kinase inhibitor; IHC, immunohistochemistry; FISH, fluorescent *in situ* hybridization; MM, microsatellite marker.

Table 1 *Patients with adrenocortical adenomas (AA)*[a]

Patient	Sex	Age (years)	Symptoms	Tumor weight (grams)	Urinary cortisol (μg/day)	ACTH (pg/ml)	17q losses
AA1	F	61	IN[b]	15	43	5	No
AA2	M	39	CS	37	544	NA	No
AA3	F	65	IN	15	56	NA	No
AA4	M	43	IN	48	59	NA	No
AA5	F	36	CS	13	961	7	No
AA6	F	81	CS	12	202	NA	No
AA7	F	69	IN	25.5	176	2	No
AA8	M	52	IN	40	92	22	No
AA9	F	46	CS	17	107	<2	No
AA10	F	30	CS	16	190	<2	Yes
AA11	F	50	IN	46	68	<2	Yes
AA12	F	56	CS	9	179	NA	No
AA13	F	20	IN	30	129	NA	No
AA14	F	54	IN	20	179	NA	No
AA15	M	32	CS	15	538	NA	No
AA16	F	36	CS		261	<5	No
AA17	F	40	CS	15	149	<10	Yes
AA18	F	32	CS	26	268	NA	Yes
AA19	F	37	IN	16	10	8	No
AA20	F	40	CS	25	194	<2	No
AA21	F	52	CS	12	26	<2	Yes
AA22	F	42	CS	9.7	265	6	No
AA23	F	62	IN	16	60	NA	Yes
AA24	M	68	IN	30	65	NA	Yes
AA25	M	41	IN	31	46	NA	No
AA26	F	44	CS	11.5	458	NA	No
AA27	F	50	IN	45	140	NA	No
AA28	F	57	CS	14	290	NA	No
AA29	F	27	CS	8	369	NA	Yes

[a] The table summarizes the main characteristics of patients with adrenal adenomas studied for allelic loss of 17q22–24 by LOH (AA1 to AA22) or FISH (AA23 to AA29) analysis. Symptoms are those at the time of diagnosis; the weight of the tumor was obtained at surgery. The normal range for urinary cortisol is <90 μg/day and for ACTH is 20–60 pg/ml.
[b] IN, incidentaloma.

MATERIALS AND METHODS

Patients and Tumor Collection. Forty-four patients with ACTs were recruited for this study (Tables 1 and 2); 29 with single adenomas [6 males (M) and 23 females (F), age 47 ± 14.1 years (mean and standard)] and 15 patients with cancer (6 M and 9 F, age 42.5 ± 14.4 years). A total of 26 patients was diagnosed with ACTH-independent CS by standard diagnostic testing. The hormonal investigations were performed as described previously (30, 31). Briefly, the diagnosis of CS was based on elevated 24-h urinary cortisol excretion (>90 μg/day), with low ACTH plasma levels (<15 pg/ml, normal range: 20–60 pg/ml), and abnormal response to either the overnight 1 mg of DXM suppression test (plasma cortisol > 40 ng/ml) and/or the classic low DXM (2 mg/day for 2 days) suppression test (urinary cortisol > 10 μg/day).

Adrenal tumors were obtained during surgery and immediately dissected by the pathologist; sections were fixed and paraffin embedded for diagnostic studies, and others were frozen and stored in liquid nitrogen until further use for the genetic studies. All adrenal tumors were examined by the same pathologist in one institution and classified by the use of widely accepted pathological criteria and molecular markers as reported previously by Gicquel *et al.* (the COMETE network; Ref. 32). Informed consent was obtained for tumor collection as part of a protocol approved by the review boards of participating institutions.

Nucleic Acid Preparation and cDNA Synthesis. DNA was extracted from peripheral blood leukocytes using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI). Tumor DNA and RNA were purified by cesium chloride gradient ultracentrifugation as reported previously (32). cDNA was synthesized by Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Groningen, the Netherlands) using 1 μg of total RNA in a final volume of 40 μl.

Table 2 *Patients with AC*[a]

Patient[b]	Sex	Age (years)	Tumor weight (grams)	Glucocorticoids	Androgens	Mineralocorticoids	ACTH	Urinary cortisol	17q losses
AC1	M	40	ND	Normal	Normal	Normal	NA	NA	Yes
AC2	F	45	284	Increased	Increased	Normal	<2	392	No
AC3	F	26	50	Increased	Increased	Normal	<2	638	No
AC4	M	52	827	Increased	Normal	Increased	<2	413	Yes
AC5	M	30	400	Increased	Normal	Normal	<2	2475	No
AC6	F	27	61	Increased	Increased	Normal	NA	110	No
AC7	F	53	104	Increased	Increased	Increased	<2	185	No
AC8	F	51	119	Increased	Increased	Normal	5	239	Yes
AC9	F	77	2600	Normal	Increased	Normal	45	80	Yes
AC10	M	72	466	Increased	Increased	Normal	<2	121	No
AC11	F	15	22	Normal	Increased	Normal	48	21	Yes
AC12	M	29	1074	Normal	Normal	Increased	14	56	Yes
AC13	F	27	570	Normal	Increased	Normal	NA	65	Yes
AC14	F	31	40	Increased	Increased	Normal	<2	3462	No
AC15	M	63	135	Increased	Normal	Normal	<2	275	Yes

[a] The table summarizes the main characteristics of patients with adrenocortical carcinomas. Allelic losses were studied by both marker and FISH analysis in seven specimens (for these samples, the final assignment of 17q status is presented); by FISH *only* in one, because the LOH analysis was uninformative (AC15) (see Table 6). For the steroids secreted by each tumor, "increased" means at least 2 SD above the mean of the respective age- and gender-specific normal range. The normal range for urinary cortisol is <90 μg/day and for ACTH is 20–60 pg/ml.
[b] For patient 1, all investigations were performed under high-dose dectancyl treatment (8 mg/day) that was prescribed for spinal metastases; in this patient, the total tumor weight is unknown because only partial resection of the primary lesion was possible.

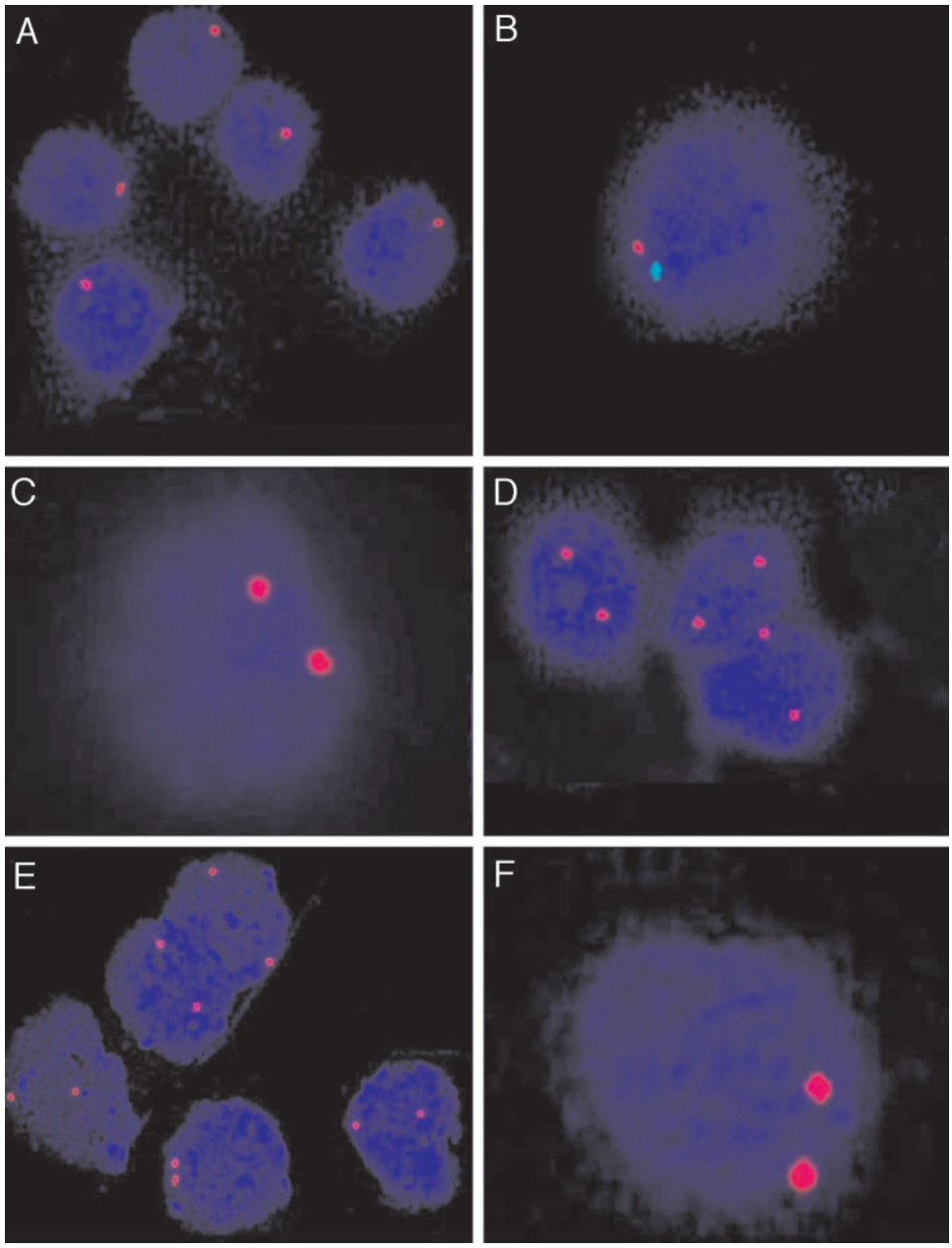


Fig. 1. Deletions of the 17q22–24 region in the adrenal tumors of the study. *A*, interphase FISH with the BAC 321-G-8 containing the *PRKARIA* gene in an adrenal adenoma showed cells with one signal of the BAC (*red*). *B*, FISH with the BAC 321-G-8 (*red*) and an α-satellite probe specific for chromosome 17 (*green*) to an adrenal carcinoma showed cells with one signal for each probe, suggesting loss of chromosome 17 from the centromere to the 17q22–24 region. *C*, a control interphase FISH for the same carcinoma with BAC RP3–526I14 from chromosome 22 showed the expected 2 signals. *D*, likewise, FISH with BAC 588-h-15 from chromosome 6 (*red*) to an adenoma that showed loss for 17q22–24 showed the expected two signals. *E*, normal adrenal cells hybridized with BAC 321-G-8 showed two copies of the probe. *F*, hybridization of BAC 321-G-8 (harboring the *PRKAR1A* gene) to another carcinoma detected two copies of the probe in all cells showing that this particular tumor had not undergone losses of the 17q22–24 region; these data were consistent with the lack of LOH (by polymorphic markers) analysis for this tumor.

FISH Analysis. Touch preparations were made from fresh or frozen tumors; these specimens were not necessarily the same as those used for the LOH or protein studies (see below), because different amounts of tissue were needed for each study. The probe that was used for FISH was a BAC that maps to 17q22–24 and contains the entire *PRKAR1A* gene (CITB BAC 321-G-8; Refs. 19, 20, and 29). Other BAC probes from chromosomes 6 and 22 were used as controls (Fig. 1). The BACs were grown, and DNA was extracted as described elsewhere (33). Probe DNA was labeled with digoxigenin-11-dUTP (Roche Diagnostics Corp., Indianapolis, IN) by nick-translation and hybridized to touch preparations of the tumors as described previously (29, 34). After hybridization, cells were counterstained with 4′,6′-diamidino-2-phenylindol-dihydrochloride. Signals for hybridization were analyzed with the use of a Leica epifluorescence microscope, and fluorescence images were automatically captured on a Photometrics-cooled CCD camera (Photometrics, Ltd., Tucson, AZ) using IP Lab Image software (Scanalytics, Inc., Fairfax, VA). At least 100 nonoverlapping cells with strong hybridization signals were scored per case. Presence of >20% cells with only one *PRKAR1A* signal was interpreted as an allelic deletion. Control touch preparations from the corresponding normal tissues showed <8% of cells with one *PRKAR1A* signal, and control probes from chromosomes 6 and 22 showed <10% of cells with one signal with no significant variance between samples and probes. A chromosome 17-specific centromeric α-satellite probe (Vysis) was used to control for polyploidy.

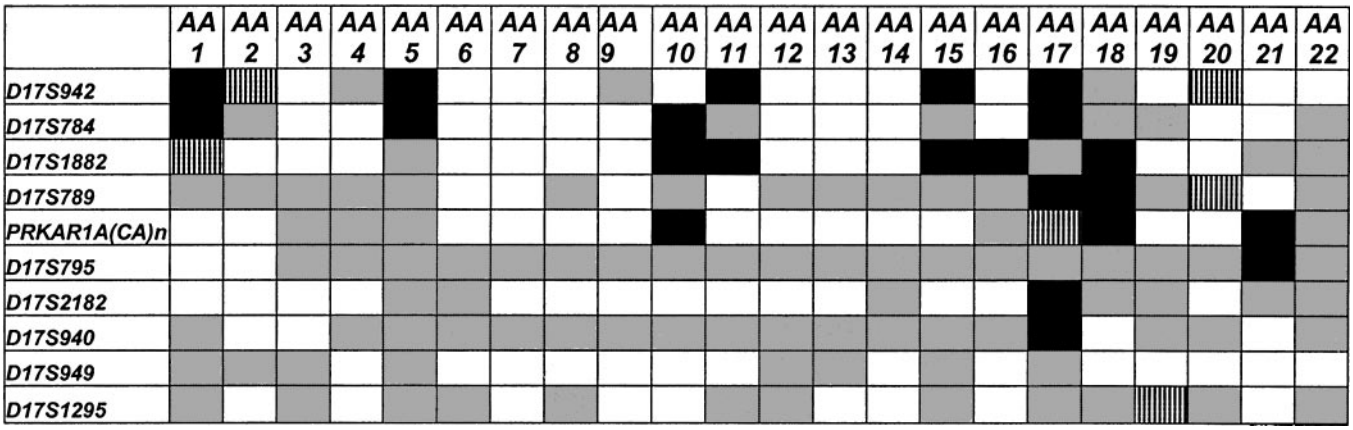
LOH Analysis. Thirty-seven paired (blood-tumor) DNA samples were studied by PCR amplification of markers surrounding the *PRKAR1A* gene (as established by contig mapping, see Ref. 19; Tables 3 and 4). The sequences and genomic order of these primers are available in the genome database online and from the Internet sites of genome centers.[5]

The tissue fragments that were used for these DNA studies were dissected from the core of the tumor mass immediately after surgical exploration and from an experienced pathologist, avoiding contamination with normal tissue.

A two-round PCR reaction was performed. In the first round, we used 10 pmol of each primer, and 200 nmol of each nucleotide (GeneAmp PCR reagent kit; Applied Biosystems, Foster City, CA). In the second round, an aliquot of the first reaction was amplified with the addition of 1 μCi of α-^{33}P-dATP

[5] Internet address: http://www-genome.wi.mit.edu.

Table 3 *LOH studies in adrenal adenomas*[a]



[a] ■, LOH; ▩, retention of heterozygosity; ▥, microsatellite instability and other size discrepancies; □, uninformative.

(Redivue deoxyadenosine 5′–[α-^{33}P]-triphosphate; Amersham Pharmacia Biotech, Piscataway, NJ). For each round, after initial denaturation (5 min at 94°C), and 35 cycles (94°C for 1 min, 57°C for 1 min, and 72°C for 3 min), samples were submitted to an extension time of 7 min at 72°C. PCR products were denaturated (94°C for 5 min), placed on ice, and loaded on a 6% nondenaturing polyacrylamide sequencing gel (Gel-Mix 6; Life Technologies, Inc., Rockville, MD) for ~2.5 h at 30 mA, depending on the size of each marker. Normal and tumor samples were loaded subsequently and in parallel to a known sequence-labeled marker to identify the exact molecular size of the bands. After electrophoresis, the gel was transferred to a filter paper and dried for 1 h in a Gel Dryer Device (Bio-Rad, Hercules, CA). The bands, corresponding to the α-^{33}P-dATP-labeled markers, were detected by autoradiography (model BioMax–MR; Eastman Kodak Film, Rochester, NY).

The microsatellite alterations seen in the tumors were classified as described previously (35). LOH was considered to be present when only one allele was evident in DNA extracted from patient's tumor compared with two alleles in the DNA derived from peripheral blood lymphocytes or if the signal from one of the two alleles had a >50% reduction in intensity, compared with that of the corresponding band in peripheral blood. Microsatellite length instability was considered to be present when the amplified tumor DNA contained multiple bands or bands that differed from those seen in DNA from peripheral blood. Specimens that were not successfully amplified were excluded from the analysis. All samples were run from preset matched wells that were prepared with automated pipetting; in addition, the results from the other markers for each pair ensured that the samples with microsatellite length instability were not mismatched. The results were expressed as a percentage of the total number of informative loci in both tumor and blood samples.

Sequencing Analysis of the *PRKAR1A* Gene. The 12 exons and flanking intronic sequences of the *PRKAR1A* gene were separately PCR amplified using the primers; the conditions were described previously for exons 1A, 1B, 2, and 7 (19, 20, 36); and the oligonucleotides are listed in Table 5 for exons 3, 4A, 4B, 5, 6, 8, 9, and 10.

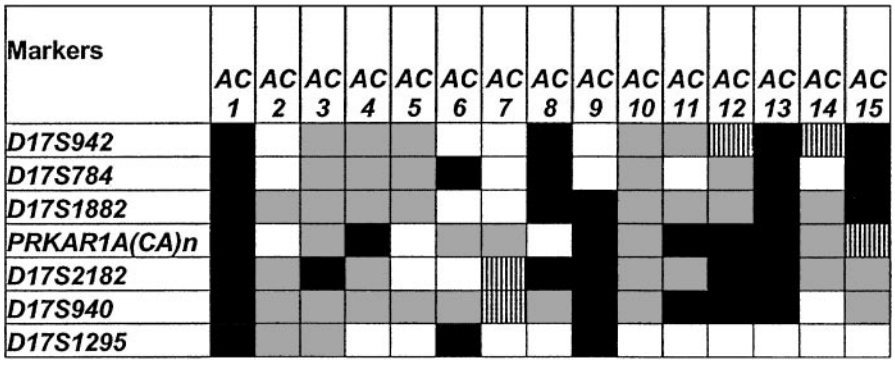
Amplified products were directly sequenced on an automated sequencer (ABI 3700; Perkin-Elmer Corp., Wellesley, MA) using the Big Dye Terminator method (Fig. 2). For samples with a mutated sequence, analysis was performed in both directions to confirm the mutation. Nucleotides were numbered in accordance with the reference sequence for *PRKAR1A* (GenBank accession no. NM_002734; Refs. 19 and 20).

Real-time PCR Quantification of mRNA. Real-time PCR was performed using the Light Cycler apparatus (Roche Diagnostics GmbH, Mannheim, Germany). For this analysis, specimens were available from 4 tumors of the 13 nonfunctioning incidentally discovered tumors ("incidentalomas" or NSAA = nonsecreting adrenal adenomas), 9 of the 13 tumors associated with CS (that did not carry mutations of the *PRKAR1A* gene; Table 1), the AA21 and AA22 tumors with *PRKAR1A*-inactivating mutations (Table 1), and 10 specimens from the 15 ACs described in Table 2.

PCR reactions were performed in a 20-μl final volume, 0.5 μM oligonucleotide, 3.5 mM MgCl2, and the Fast Start DNA master SYBR Green (Roche) according to the manufacturer recommendations. Primers used for *PRKAR1A* and *GAPDH* amplification are shown in Table 5. Annealing was performed at 56°C for *PRKAR1A* amplification and 58°C for *GAPDH*. Product specificity was controlled by melting curve analysis and migration on a 1% agarose gel. Results are analyzed with the software of the Light Cycler apparatus. Standard curve is determined by the use of a plasmid containing the PCR product amplified from cDNA made from RNA extracted from the H293 cell line and cloned in pGEM-T easy, as reported previously (36).

Preparation of Protein Lysates and Western Blotting. To prepare total protein extracts, additional large segments of frozen tissue were homogenized in PBS; homogenates were resuspended in three volumes of M-PER reagent

Table 4 *LOH studies in adrenal cancer*[a]



[a] ■, LOH; ▩, retention of heterozygosity; ▥, microsatellite instability and other size discrepancies; □, uninformative.

Table 5 *PRKAR1A oligonucleotide sequences for mutation detection*

Exon 3	
Sense	5′-GAATTGGTGTTTTCCTCTTAACTT-3′
Antisense	5′-TATGATTCATTCATCAAAGGAGAC-3′
Exon 4A	
Sense	5′-AATGTTTTTGGTTTATGGAATTGT-3′
Antisense	5′-CACACCCTTACTTGAAAAATAGTG-3′
Exon 4B	
Sense	5′-GACAGTCTGGGGTCTTTAATTCTA-3′
Antisense	5′-TCAAAGAGGAAAACAAACTTCAAT-3′
Exon 5	
Sense	5′-TTTCTTTAATTTGGAATATGCTTC-3′
Antisense	5′-ATCTGACATACAAGGGATGTAATG-3′
Exon 6	
Sense	5′-TTTTTAAAACAAAGTTCAGGATTG-3′
Antisense	5′-CTAAATCACACTCTCAAACACCAT-3′
Exon 8	
Sense	5′-GGCTATTTGGTTGAATCTCTTTAT-3′
Antisense	5′-TGAGTTCTTTACCTCTAAAATTCAA-3′
Exon 9	
Sense	5′-TTGTTTAGCTTTTTGGTGATTTTA-3′
Antisense	5′-GGAGAAGACAAAATTATGGAAGAC-3′
Exon 10	
Sense	5′-TATTGTCTTCTTTCTCAGAAGTGC-3′
Antisense	5′-GTGCAATAAAAGCAACTTTCAATA-3′
For cDNA and real-time PCR studies	
PRKAR1A	
Sense	5′-AAT GGC CGC TTT AGC CAA AGC C
Antisense	5′-TTC TCC AAA GCT CCC TCC TTC
GAPDH	
Sense	5′-GCC ACA TCG CTC AGA CAC CA
Antisense	5′-TTC CCG TTC TCA GCC TTG AC

supplemented with complete protease inhibitor cocktail (Roche Biochemicals, Inc., Indianapolis, IN). Protein concentrations were determined by the bicinchoninic acid assay kit (Pierce, Rockford, IL), and 50 mg of protein were resolved in a 4–12% NuPage gel in 4-morpholineethanesulfonic acid buffer (Invitrogen, Carlsbad, CA) before transfer to polyvinylidene difluoride membranes. Western blots were performed using the Western Breeze kit for murine antibodies as directed by the manufacturer (Invitrogen). Monoclonal antibodies specific for R1α, R2α, R2β, and Cα were obtained from BD Transduction Laboratories (Lexington, KY) and used at dilutions specified by the manufacturer, as described elsewhere (20, 29).

To compare R1α protein levels in tumors with and without *PRKAR1A* mutations and/or 17q losses in the same experiment, protein was extracted anew from the three tumors with mutations (AA6, AA21, and AA22) and three without *PRKAR1A*/17q genetic changes (AA7 and two additional "control" adrenal tumors that were sequenced and checked for 17 q losses and had none). The lysates were processed, and the Western blots were performed as above. After exposure, the films were scanned, and absorbance was recorded by standard methods.

IHC. Sections from paraffin-embedded tissue from the two tumors with *PRKAR1A* mutations (AA21 and AA22), two tumors with LOH for 17q22–24 but without *PRKAR1A* mutations (AA 10 and 18), and three tumors without mutations or 17q losses (AA7 and the two additional tumors referred to above) were processed for IHC with monoclonal antibodies specific for RIα and the other main PKA subunits (RIIα, RIIβ, and Cα) under conditions specified by the manufacturer (BD Transduction Laboratories, San Diego, CA). At least two blinded readers graded the specimens for all stainings, as reported elsewhere (29). Briefly, the specimens were graded 0–2 with 0 being no different from the negative control (serial section of the same specimen processed for IHC without the specific antibody), 1 showing moderate expression and 2 reflecting strong immunoreactivity.

Determination of PKA Activity. Kinase activity was measured, as described previously (19, 29), in cpm using γ-^{32}P-dATP (deoxyadenosine 5′-[γ-^{32}P]-triphosphate; Amersham Pharmacia Biotech), in 2 mg of total protein cell extracts from tumors that had been snap frozen in liquid nitrogen at the time of their excision. All determinations of PKA activity were done twice for each tumor, corrected for protein content, and an average value was calculated for each experiment.

Total kinase activity represents enzymatic activity after stimulation with cAMP; total PKA-specific activity represents the difference between PKA activity before and after the addition of PKI, as described previously (19, 29). Free PKA activity was calculated also as described previously (29); a ratio was then calculated according to the following formula [both total and free PKA activity are expressed in units (U) per milligram of protein (units/mg)]:

PKA activity ratio = Free PKA activity (in units/mg protein content)/Total PKA activity (in units/mg protein content)

Data from all tumors were compared with STATISTICA software (StatSoft, Inc., Cary, NC) using the *t* test for individual comparisons between the two diagnostic groups [carcinomas ($n = 8$) and adenomas ($n = 7$)]. A $P < 0.05$ was considered to indicate significance; a $P < 0.1$ was interpreted as showing a tendency toward a statistically significant difference.

Other Statistical Analyses. All data are shown with the mean ± SE, unless indicated otherwise. Statistical analysis for quantitative PCR data were performed by nonparametric test (Mann-Whitney) using the StatView 5.0

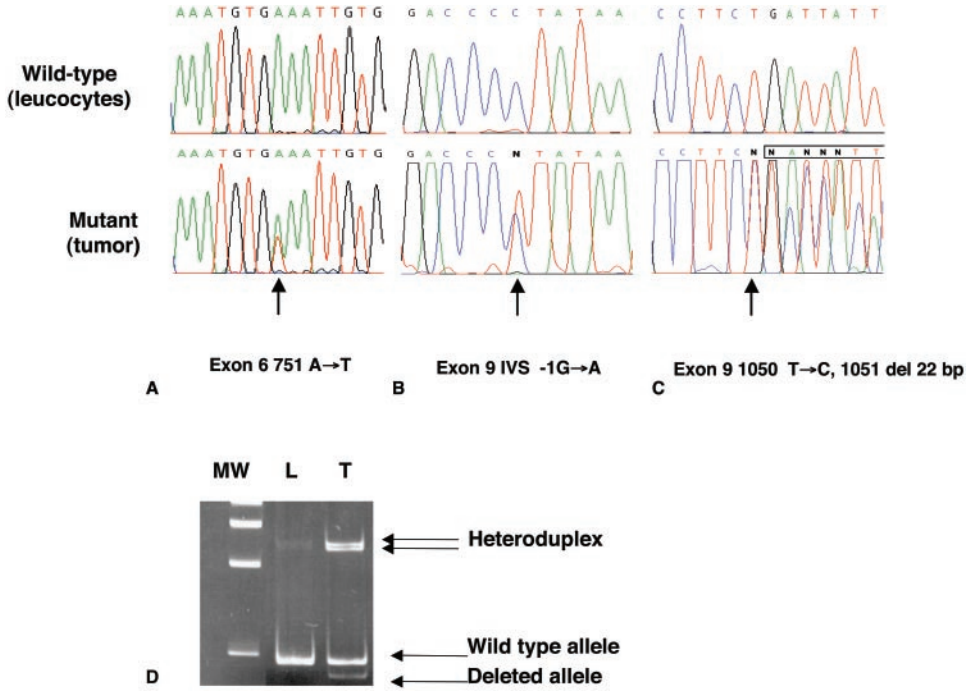


Fig. 2. Sequencing of the three tumors with *PRKAR1A* mutations. *A–C*, the sequencing of the leukocyte DNA (*top*) and tumoral DNA (*bottom*) of the three adrenal adenomas (*AA*) with a somatic *PRKAR1A* mutation. Sequencing was performed in both strands as described in "Materials and Methods." The sequence of the sense strand is shown in *A* and *C*, whereas that of the complementary strand is shown in *B*. *A*, the nonsense mutation 751 A > T found in AA6; *B*, the splice site mutation 9IVS –1G < A found in AA21; *C*, the exon 9 point mutation and subsequent deletion found in AA22 (1050 T > C, 1051 del 22 bp). *D*, a nondenaturing polyacrylamide gel of PCR amplicons of exon 9 from leukocyte (*L*) and tumoral DNA (*T*); the heterozygous 22-bp deletion and related heteroduplex were present only in the tumor, as it would be expected from a somatic alteration.

Table 6 *Comparison of 17q FISH and LOH studies in adrenal cancer specimens*[a]

Adrenal cancer specimen[b]	LOH analysis by MM	FISH by BAC 321G8	Final classification
AC5	No LOH	No loss (10%)	No 17q losses
AC6**	No LOH	Loss (100%)	17q loss
AC7	No LOH	No loss/polyploid	No 17q losses
AC8	No LOH	Loss (70%)	17q loss
AC9**	LOH	Loss (29%)	17q loss
AC10	No LOH	No loss (10%)	No 17q losses
AC11	LOH	Loss (85%)	17q loss
AC15**	LOH/uninformative	No loss (16%)/polyploid	17q loss

[a] In this Table, LOH refers to loss of the intragenic *PRKAR1A(CA)n* marker (see also Table 5), which is located in the 5′-(promoter) region of the *PRKAR1A* gene (19). BAC321G8 contains part of the *PRKAR1A* gene, everything after exon 2 to its 3′-region (19, 20). In the third column, the number in parenthesis represents the percentage of cells that was scored for the presence of a signal specific for the probe used (see "Materials and Methods" and also Fig. 1). The cut off for considering a FISH study "positive" for 17q loss was 20%, as commonly used (37, 38), although most specimens with losses had significantly more than that in our study; the three that did not are indicated by the two asterisks (**) and are discussed in the text. Polyploidy was evident if in most cells, more than two signals for each probe were seen.

[b] The numbering is the same as that used in Tables 2 and 4 for all AC specimens; in Table 2, the last column (17q losses) represents the final status that was used for all analyses and corresponds to the fourth column in this table for the specimens that underwent *both* FISH and LOH studies.

program (SAS Institute); significance was set at $P < 0.05$. The χ^2 test was used to compare rates of LOH or mutations between adenomas and cancer, responses to DXM 150% over baseline and higher, and tumor weights < 15 grams (with the Fischer correction, when needed); PKA activity comparisons between adenomas with and without LOH and cancers with and without LOH were done with the *t* test, as described above.

RESULTS

Mapping of the *PRKAR1A* Gene. We have mapped previously the *PRKAR1A* gene to the chromosomal region 17q22–24 by sequence tag (STS)-mapping to a known contig of the area (19) and were able to use polymorphic markers from the region for LOH studies. We also mapped a BAC containing the *PRKAR1A* gene (BAC 321-G-8) by FISH to the 17q22–24 region and determined its position with 10 linked MMs in a 10 cM area flanked by *D17S942* and *D17S1295* on 17q22–24 (data not shown). Among them, 8 MMs surrounding (and one from within) the *PRKAR1A* gene, were chosen for the LOH studies. The 321-G-8 BAC was used for FISH analysis of frozen tumor preparations.

17q Allelic Studies in Adenomas. We studied a total of 44 ACTs from 29 patients with single adenomas and 15 patients with cancer; their clinical data and 17q22–24 molecular cytogenetic results are presented in Tables 1 and 2.

Allelic losses by FISH were seen in two of seven adenomas (Fig. 1) for which either DNA from normal tissue was not available or MM analysis was uninformative (Table 1). LOH by MM analysis was detected in 5 of 22 adenomas (23%) for which paired blood and tumor DNA samples were available and the studies were informative (Table 3).

17q Allelic Studies in Carcinomas. Allelic losses by FISH were seen 6 of 8 cancers (compared with two of the seven adenomas; $P = 0.1$; Fig. 1). LOH by MM analysis was detected in 8 of 15 cancers (53%; Table 4), a difference with adenomas that was significant ($P = 0.039$).

In some carcinomas (from which adequate sample was available because of their large size; $n = 8$), we obtained both LOH and FISH studies (Table 6). In five tumors, the two studies produced identical results (63%); in three, the data were discrepant, and after reanalysis of the touch preparations and LOH studies, as it has been suggested elsewhere (37), we assigned their status for further analysis, based on several factors that are described below:

For AC6, all cells tested by FISH showed loss of one signal; *PRKAR1A (CA)n*, however, did not show LOH, and the next marker (*D17S1882*) was uninformative, whereas the very next (*D17S784*) showed LOH. We interpreted these data as consistent with 17q loss, most likely a deletion of one *PRKAR1A* allele that occurred after exon 1 of the gene.

For AC9, all tested markers showed LOH (with the exception of the two centromeric sequences *D17S784* and *D17S942*, which are located at least 2 million bp away from the *PRKAR1A* gene and were uninformative; see Table 4). FISH in this specimen showed 29% of the cells (only 9% above the cutoff of 20%) with deletion, which was the lowest percentage among all our FISH-positive tumors (Table 6). This was a large carcinoma (see Table 2) with large segments of the specimen "contaminated" with normal cells, which may explain the lower percentage of cells with deletions in the FISH studies.

Finally, AC15 had LOH for all centromeric markers up to *PRKAR1A(CA)n*, which, however, was uninformative because of microsatellite instability (Table 4); the FISH studies were difficult to

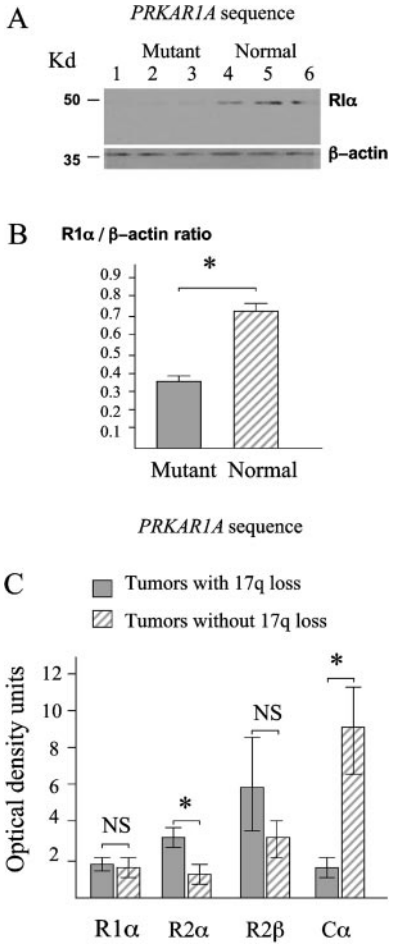


Fig. 3. Western blotting of tumors with *PRKAR1A* mutations or 17q22–24 losses. *A*, R1α and β-actin levels in protein lysates from the three tumors with *PRKAR1A*-inactivating mutations (AA6, AA22, and AA21 in *Lanes 1–3*, respectively) and three adrenal adenomas without 17q22–24 LOH or *PRKAR1A* mutations (AA7 and two additional tumors *in Lanes 4–6*, respectively). *B*, the R1α:β-actin ratio, in arbitrary absorbance units, in the three tumors from *Lanes 1–3 versus* that of the three tumors in *Lanes 4–6*, a difference that is significant (*P, < 0.05). *C*, Western blots for the main PKA regulatory subunits and the Cα catalytic subunit in tumors with ($n = 4$) and without 17q allelic losses ($n = 3$). The data represent arbitrary absorbance units (as in *B*). There is no difference in the R1α content, as detected by immunoblotting, but the overall type I:type II PKA subunits ratio is altered significantly in tumors with 17q losses. This finding is associated with the significant difference of the Cα content of these tumors, which is shown here and similar to Cα changes in other experimental settings with type I *versus* type II PKA alterations (see "Discussion").

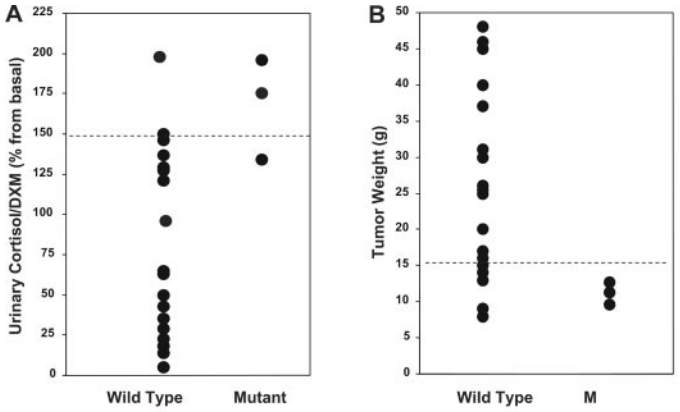


Fig. 4. Response to DXM and tumor weight in the adrenal adenomas with and without *PRKAR1A* somatic mutation. *A*, the 24-h urinary free cortisol response to DXM (2 mg/day) expressed as an increase in percentage from the baseline in patients with CS ($n = 26$), from the adenomas harboring *PRKAR1A* mutations ($n = 3$) on the *right*, and from the tumors without mutations ($n = 23$) on the *left*. *Dashed line*, the cutoff value of 150% used for statistical analysis. *B*, the weight of the adrenal adenomas with *PRKAR1A* somatic mutations ($n = 3$) on the *right* and of the adenomas without mutations on the *left* ($n = 26$). *Dashed line*, the cutoff value of 15 grams that was used for statistical analysis.

interpret because most cells had more than four signals, indicating polyploidy and, perhaps, explaining the instability seen at the microsatellite level (Table 6). Given the LOH studies, this tumor, too, was interpreted as having 17q losses.

Sequencing Analysis of the *PRKAR1A* Gene and Clinical Correlation. Sequencing of the 12 exons of *PRKAR1A* was performed in tumoral DNA extracted from all 44 ACTs. Three different genetic changes were found in three adrenal adenomas (AA): (*a*) a nonsense mutation (751 A>T in exon 6) was found in AA6; (*b*) a splice site mutation (9IVS-1 G>A) was found in AA21; and (*c*) a point mutation (1050 T>C) followed by a 22-bp deletion in exon 9 of the gene was observed in AA22 (Fig. 2). These mutations were not observed in the leukocytes of these patients, a finding consistent with somatic alterations. All of these changes lead to predicted shorter R1α protein products; however, the shorter protein was not detected in Western blotting with an R1α-specific antibody (Fig. 3*A*), indicating that they, most likely, lead to nonsense mRNA decay, as we have shown to be the case in other *PRKAR1A*-inactivating mutations (19, 20, 39). The same antibody detects shorter R1α protein products made of the mutant gene after *in vitro* translation (19, 20).

It is noteworthy that these three *PRKAR1A* mutations are novel; they are not among those that we (19, 20, 29, 36, 39) and others (40) have described in CNC patients. These patients were screened extensively and did not meet the criteria for CNC (28), and their mutations were somatic (Fig. 2). Interestingly, the three adenomas that harbored these mutations were responsible each for CS and appeared pigmented on pathological examination, although the cortex had no other features of PPNAD (data not shown). Cyclic hypercortisolism, a frequent feature of PPNAD in the context of CNC (26, 27), was also observed in patient AA21; this patient had alternating periods of high and low cortisol excretion associated with ACTH-independent CS and adrenocortical insufficiency, respectively, during the 1 year that she was followed preoperatively.

An additional clinical feature that the 3 patients with the adenomas that harbored *PRKAR1A* mutations all shared was a paradoxical increase of cortisol secretion during DXM administration (Fig. 4*A*). This response is considered diagnostic for PPNAD in the presence of bilateral adrenocortical hyperplasia; in a recent study, only 15% of sporadic adrenocortical adenomas had low level glucocorticoid rise in response to DXM (27). Indeed, in the present study, such paradoxical responses were only rarely present in the adenomas without *PRKAR1A* mutations; of a total of 18 studied, 15 had cortisol responses <150% from the baseline, whereas two of the three adenomas with mutations had a cortisol rise over 160%, and the third had a rise of 130% (Fig. 4*A*; $P = 0.02$). Some of the tumors with 17q losses had some increase of cortisol excretion over the baseline in response to DXM, which, however, was neither significant nor more frequent than that seen in sporadic adrenal adenomas or cancer (27).

Another feature shared between known PPNAD characteristics (21, 22, 27) and those of the adenomas bearing *PRKAR1A* mutations was the relatively small size of the latter. The average weight of the three adenomas with mutations was 11.2 $\pm$ 0.8 grams; only two of the other adenomas were <15 grams ($P = 0.007$), and their average weight was 23.4 $\pm$ 12.05 grams (Table 1; Fig. 4*B*).

It is noteworthy that none of the 15 cancers had mutations of the *PRKAR1A* gene (data not shown), a difference from adenomas that was, however, not significant ($P = 0.4292$).

mRNA and Protein Studies (Western Blotting and IHC). Quantitative, real-time PCR demonstrated variable levels of *PRKAR1A* among ACTs. All experiments were done in duplicate, however, and the results were almost identical for each specimen, despite the interspecimen variability (data not shown). For all of the tumors that were studied, the data were expressed as the number of copies of *PRKAR1A* mRNA *versus* the number of copies of the housekeeping *GADPH* mRNA, as explained in "Materials and Methods." These data are shown in Fig. 5. Enough material to study *PRKAR1A* expression by real-time PCR was available from two of the adenomas with mutations (AA21 and AA22). Among the nonmutant tumors, nonsecreting adenomas had the highest level of *PRKAR1A* mRNA (0.232 $\pm$ 0.098 copies of *PRKAR1A* corrected for *GAPDH*), when compared with adrenal adenomas responsible for CS (0.034 $\pm$ 0.009) and cancers (0.004 $\pm$ 0.002; $P < 0.001$; Fig. 5). The two adenomas with *PRKAR1A* mutations exhibited a very low level of *PRKAR1A* mRNA (0.001 $\pm$ 0.0000245) that was comparable with that of adrenal tissue from patients with germ-line *PRKAR1A*-inactivating mutations (data not shown).

Western blotting with a R1α-specific antibody showed differences consistent with the mRNA studies; R1α protein was decreased in the three tumors (AA6, AA21, and AA22) with *PRKAR1A* mutations when compared with tumors that had no mutations or 17q genetic changes (Fig. 3, *A* and *B*).

The findings on IHC (with the same antibodies that were used

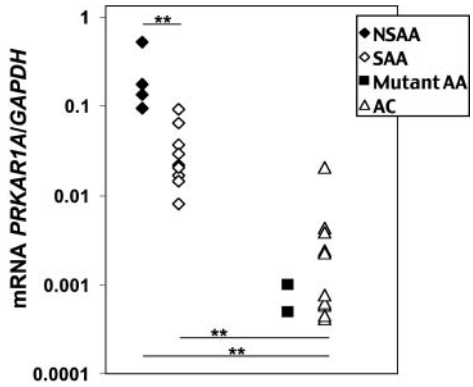


Fig. 5. Quantification of *PRKAR1A* mRNA in adrenocortical tumors. The figure shows the results of *PRKAR1A* mRNA quantification as determined by real-time PCR using a light cycler (see "Materials and Methods"). Results are expressed as copies of *PRKAR1A* corrected for *GAPDH*. NSAA ($n = 4$) are the nonsecreting adrenal adenomas, SAA are the secreting adrenal adenomas (those responsible for CS), mutant AA are the adrenal adenomas AA21 and AA22 with somatic *PRKAR1A* mutations, and AC are the adrenocortical cancers. **, a $P < 0.01$. Similar results were obtained in two independent experiments.

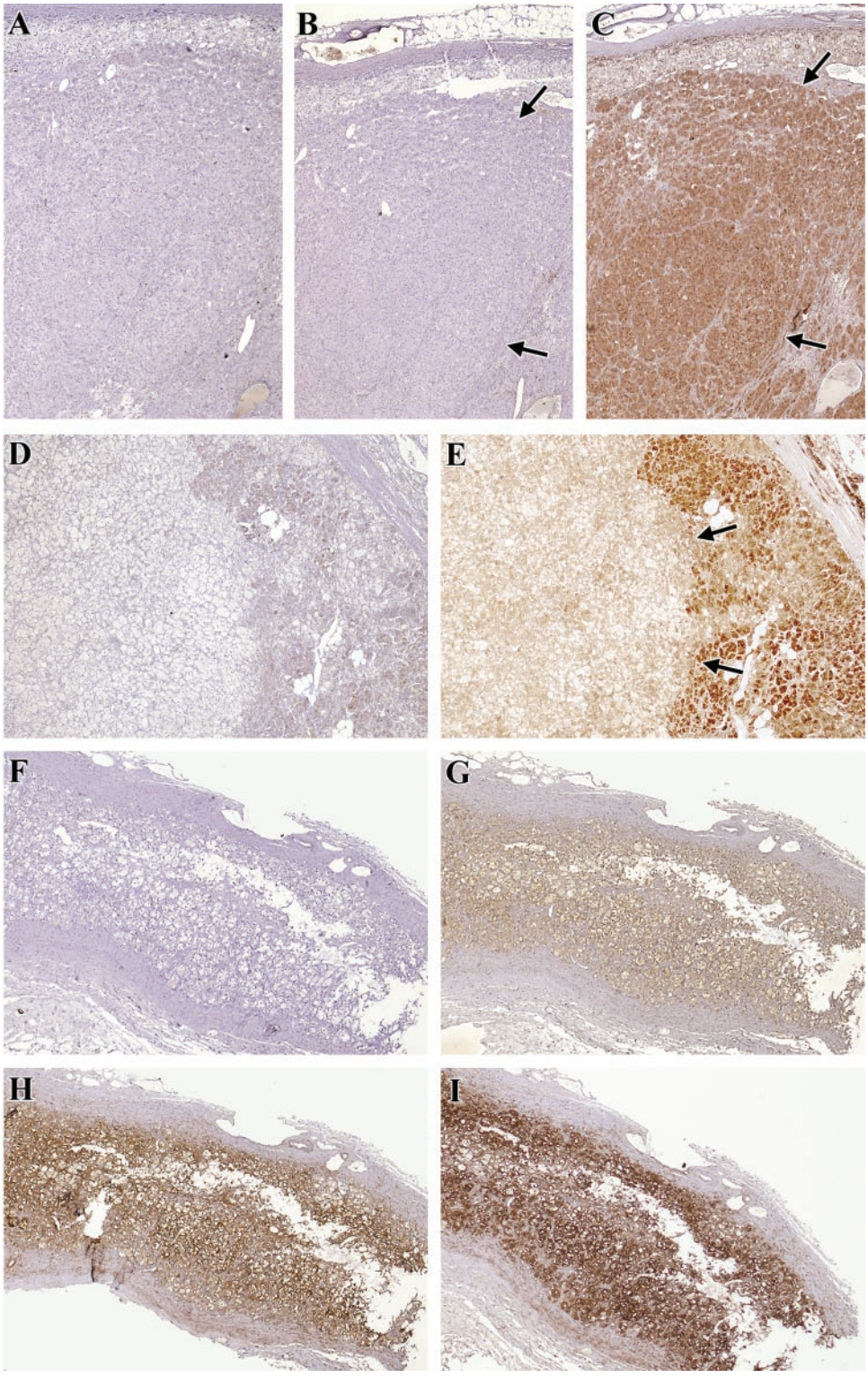


Fig. 6. IHC for PKA subunits in tumors with and without PRKAR1A or 17q22–24 genetic changes. *Top panels*, the staining without a specific antibody (negative control; *A*) and with antibodies specific for R1α (*B*) and R2β (*C*) of the AA22 tumor, which did not have 17q allelic losses but had a *PRKAR1A*-inactivating mutation and down-regulation of its message and protein products (see Figs. 3 and 5); in the tumor cells (indicated by the *arrows*), there is very little, if any, staining for R1α, but there is strong immunoreactivity for R2β. *D* (negative control) and *E* show immunostaining of an adenoma (AA18) with LOH for 17q22–24 markers and no coding sequencing defects of the *PRKAR1A* gene, which showed decreased R1α expression within the tumor (*arrows*) compared with surrounding adrenocortical cells that are normally stained. The remaining *panels* show the staining of a hyperplastic nodule of tumor AA7, which did not have *PRKAR1A* mutations or any 17q allelic losses, without a specific antibody (negative control; *F*), and, then, with antibodies specific for R1α (*G*), R2α (*H*), and R2β (*I*). There is immunoreactivity for all three PKA subunits.

above) correlated well with those of Western blotting. Consistent with what is shown in Fig. 3*A*, there was no detectable expression of the R1α protein in AA21 and AA22 (Fig. 6*B*) compared with that of adenoma AA7 or other nodular adrenal tissue that did not harbor any 17q genetic changes or *PRKAR1A* mutations (Fig. 6*G*). Interestingly, too, adenomas with LOH for 17q22–24 showed lower R1α expression in adenomatous tissue compared with that in surrounding normal adrenal cortex; a representative staining is shown in Fig. 6*E* for AA18, which had LOH from *D17S1882* to the *PRKAR1A* locus (Table 3), but did not have *PRKAR1A* mutations.

When all of the PKA subunits were studied by Western blotting, among tumors without R1α mutations, in these samples with LOH and/or other allelic 17q losses, there was higher R2α ($P = 0.027$) but not R2β ($P = 0.48$), and a significantly lower Cα ($P < 0.001$; Fig. 3*C*); the R1α levels *per se* were not significantly different ($P = 0.67$). Overall, although tumors without 17q losses had a R1α:R2α ratio of

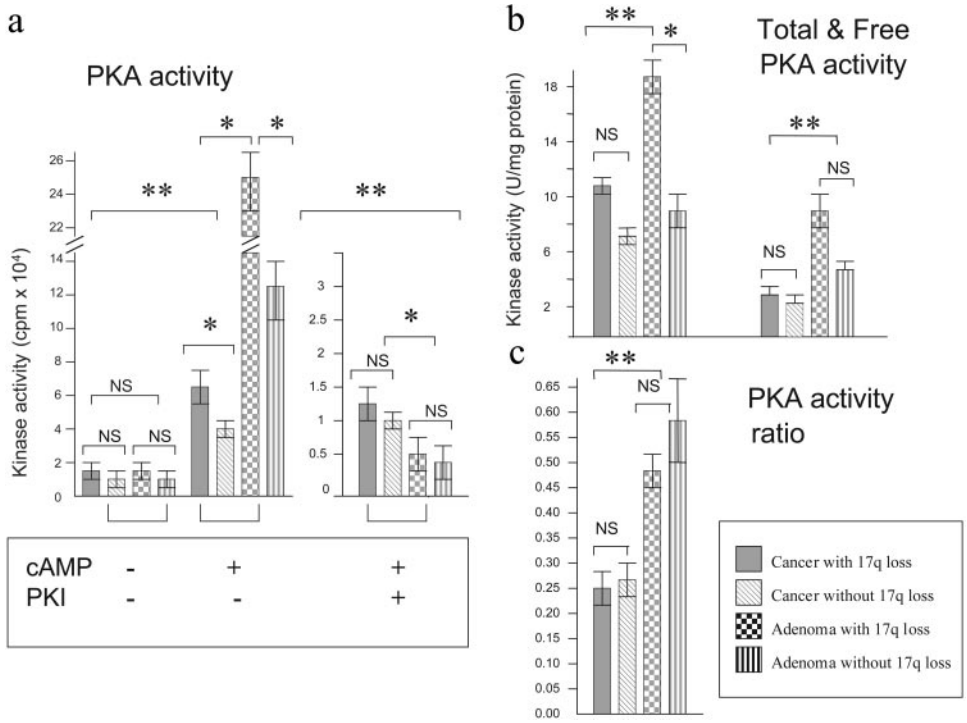


Fig. 7. Protein kinase A activity in cancer and adenomas. PKA activity in cancer ($n = 8$) with and without losses of the 17q22–24 region ($n = 5$ and 3, respectively) and in adenomas ($n = 7$) with and without allelic losses ($n = 3$ and 4, respectively). *a*, PKA activity at baseline, after exposure to cAMP and inhibition with PKI; adenomas have higher PKA activity in response to cAMP, but cancers have greater residual activity after the application of PKI (see "Results" and "Discussion"). *b*, total (on the *right*) and free PKA (on the *left*) activity; both are higher in adenomas (see text). *c*, PKA activity ratio (see "Materials and Methods") is lower in cancer than in adenomas (see text).

0.53 ± 0.007, tumors with 17q losses had a ratio of 1.21 ± 0.08 ($P < 0.001$). Accordingly, the former had a total R subunit (R1α + R2α + R2β) to Cα ratio of 0.72 ± 0.0001, whereas in the latter, this ratio was 6.39 ± 1.05, a significant difference ($P = 0.023$). IHC supported these data; adenomas with *PRKAR1A* mutations or 17q losses tended to have strong immunoreactivity for type-II subunits (Fig. 6*C*).

Protein Kinase A Activity. At baseline, all groups of tumors that were studied [a total of eight cancers with (group 1, $n = 5$) and without 17q allelic losses (group 2, $n = 3$) and 7 adenomas with (group 3, $n = 4$) and without 17q allelic losses (group 4, $n = 3$)] had similar kinase activity (Fig. 7*a*): group 1 (17,565 ± 2,763 cpm), group 2 (13,288 ± 1,660 cpm), group 3 (17,985 ± 2,368 cpm), and group 4 (12,050 ± 2,097 cpm; for all comparisons, $P > 0.05$).

After exposure to cAMP, all tumors responded with an increase in kinase activity ($P < 0.001$, for all comparisons between each group's baseline and after cAMP-peak value; Fig. 7*a*). However, adenomas overall had a much higher stimulation of their kinase activity (198,806 ± 27,915 cpm) than cancers (56,836 ± 4,474 cpm; $P < 0.001$). The opposite was true for inhibition of the cAMP-stimulated activity by PKI, a PKA-specific inhibitor: cancers were inhibited less (12,515 ± 1,544) than adenomas (5,645 ± 1,364; $P = 0.006$).

Both cancers and adenomas with 17q allelic losses had higher stimulation of kinase activity in response to cAMP *versus* that without 17q losses: group 1 (63,386 ± 5,163 cpm) *versus* group 2 (45,920 ± 1,646 cpm; $P = 0.046$) and group 3 (250,597 ± 20,370 cpm) *versus* group 4 (129,752 ± 21,277 cpm; $P = 0.009$). There were no statistically significant differences between each subgroup after inhibition with PKI: group 1 (13,501 ± 2,294 cpm) *versus* group 2 (10,871 ± 1,600 cpm; $P = 0.45$) and group 3 (6,014 ± 1,788 cpm) *versus* group 4 (5,154 ± 2,535 cpm; $P = 0.78$).

Adenomas had higher total (14,486 ± 2,064 units/mg) and free PKA activity than cancers (9,433 ± 729 units/mg; Fig. 7*b*). Both total and free PKA activity were higher in adenomas with 17q losses (18,343 ± 1,520 and 8,977 ± 1,363 units/mg, respectively) than those without (9,344 ± 1,437 and 5,172 ± 613 units/mg, respectively), although the free PKA comparisons did not reach significance levels ($P = 0.009$ and 0.074, respectively). Between cancers with and without 17q losses, there was a tendency for higher total PKA activity in the former (10,457 ± 866 units/mg) than latter (7,726 ± 367 units/mg; $P = 0.06$); free PKA activity was not different between the two groups (2,666 ± 465 and 2,056 ± 312 units/mg, respectively; $P = 0.39$).

PKA activity ratio was higher in adenomas than carcinomas (0.529 ± 0.057 *versus* 0.259 ± 0.024, respectively; $P < 0.001$; Fig. 7*c*). This ratio was not significantly lower in adenomas with 17q losses (0.486 ± 0.054) *versus* those without losses (0.586 ± 0.118; $P = 0.43$); it was also not different between cancers with (0.255 ± 0.036) and without 17q losses (0.266 ± 0.037; $P = 0.84$).

DISCUSSION

Several genetic abnormalities have been described in adrenal tumors (9, 10). In general, adrenal adenomas tend to have fewer genetic changes than cancers (1, 4, 32, 40); the extent of their genetic alteration correlates positively with size, which in ACTs is a reliable indicator of the benign or malignant nature of the tumor (41, 42). In the present study, we investigated the hypothesis that sporadic ACTs had somatic genetic changes of the 17q22–24 locus and/or the *PRKAR1A* gene. A recent study by comparative genomic hybridization, a technique with significantly less sensitivity than FISH and LOH (43), found 17q alterations in ACTs (44). Zhao *et al.* found gains of the most telomeric portion of 17q, an area that is distant from the *PRKAR1A* locus; other data indicate that more centromeric regions are likely to be involved in deletions (42). Our FISH and LOH data indeed confirmed frequent losses of the 17q22–24 region in ACTs; again, carcinomas had more frequent losses than adenomas (Tables 3 and 4).

This is the only study, to our knowledge, that has examined by both FISH and LOH several adrenal tumors. The two investigations produced similar results in 63% of the specimens, a rate that is generally similar to that in other settings, *e.g.*, bladder cancer (38) or endocrine

pancreatic tumors (45). In our reanalysis, factors such as the amount of tissue available (whether the touch preparation was done from the center of the mass or its edges), the cells that were found monosomic and/or polyploid by FISH, the benign or malignant nature of the tumor, as well as whether LOH was present in other, proximal MMs were taken into account (Table 6).

Several lines of evidence suggest that these changes are specifically related to *PRKAR1A*: (*a*) in addition to 17q BAC losses and LOH of several MMs, we also studied an intragenic *PRKAR1A* marker (19, 20); (*b*) unlike the widely present correlation between the size of ACTs and number of their genetic defects (41, 42), we found no correlation between the size of our tumors and frequency or extent of 17q losses; and (*c*) perhaps most importantly, sequencing of all ACTs in this study showed that three adenomas from unrelated patients with CS but without CNC or any other inherited condition harbored somatic *PRKAR1A*-inactivating mutations. These tumors were the smallest in size and associated with paradoxical cortisol responses to DXM (Fig. 4), all features of PPNAD (26–28).

It should be noted, however, that in two of the three tumors with *PRKAR1A* mutations (patients AA6 and AA22), we were not able to show losses of the *PRKAR1A* locus with the caveats that, at least, in patient AA6, few of the studied MMs were informative (Table 3) and that we did not use microdissected specimens for our FISH and DNA studies, which makes "contamination" with normal cells from these benign tumors not unlikely. *PRKAR1A*'s complex molecular regulation and possible methylation of its promoter[6] may also account for the lack of LOH in these specimens, as well as for the absence of *PRKAR1A* coding sequence mutations in the DNA of the other tumors with 17q allelic losses. Indeed, down-regulation of the R1α protein was suggested by immunostaining in two specimens with 17q losses and lack of mutations of the coding sequence of the *PRKAR1A* gene (Fig. 6*E*). In at least one other example of a tumor suppressor gene involved in endocrine tumorigenesis, methylation of the *PTEN* promoter was the apparent explanation behind the discrepancy between high rates of 10q LOH and absence of detectable sequence alterations in thyroid tumors (46). Furthermore, more recent data from our laboratory (39) also support the notion that *PRKAR1A* haploinsufficiency may be sufficient for tumorigenesis in at least some cases. According to these data, *PRKAR1A* may cause tumors even after simple haploinsufficiency is caused by either mutations of epigenetic silencing from one allele, whereas loss of the normal allele may further exacerbate its tumorigenic activity by annihilating any PRKAR1A expression in certain tissues or cells.

Could the lack of *PRKAR1A* mutations in tumors with 17q allelic losses indicate presence of another tumor suppressor gene in its proximity? This was the case with at least one other gene near *PTEN* at 10q; the identification of *MINPP1* showed that at least some thyroid tumors with 10q LOH (47) had mutations of this gene (and not of *PTEN*; Ref. 48). However, LOH around *PTEN* involved genomic regions significantly larger (47) than the *PRKAR1A* locus, and examination of the available 17q22–24 genomic maps between *D17S942* and *D17S1295* did not show any other expressed sequences likely to be functioning as tumor suppressor genes (19, 20).

In favor of *PRKAR1A*'s specific involvement in the tumors included in this report are three additional studies that we performed: (*a*) quantitative PCR showed down-regulation of the *PRKAR1A* mRNA in tumors with inactivating mutations and adenomas with 17q allelic losses only (Fig. 5); (*b*) Western blotting showed that tumors with 17q losses had more type II regulatory subunits than tumors without any 17q changes (Fig. 3), just as in PPNAD (49) and cultured fibroblasts (40) carrying *PRKAR1A* inactivating mutations. A switch to type II PKA has been postulated as the underlying anomaly in cAMP signaling in CNC tumor cells (19, 20, 40, 50). Increased stability of mRNA or protein, and in some cases transcriptional enhancement, may be responsible for the increased presence of type-II regulatory subunits in these tumors (51–55); and (*c*) we found significant PKA activity alterations between tumors with and without 17q allelic losses and between cancers and adenomas. In general, loss of 17q22–24 alleles was associated with higher response of total kinase activity in response to cAMP and relatively decreased PKA activity ratio (Fig. 7). We have reported both these features in tumors associated with CNC and carrying PPNAD-inactivating mutations and 17q22–24 allelic losses (19, 22), as well as in sporadic thyroid carcinomas with somatic *PRKAR1A* down-regulation (29). Although regulation of the PKA tetramer differs significantly between tissues and cells at different developmental stages (56), these data are also consistent with the lower kinase activity found in RII$\beta-/-$ mouse cells that have a compensatory up-regulation of RIα (57, 58).

A significant difference between the results of this study and our previous one on sporadic thyroid tumors (29) is the greater cAMP-induced PKA activity in adrenocortical adenomas; thyroid adenomas, to the contrary, had lower kinase activity than undifferentiated (anaplastic) carcinomas (29). The present study also showed that adrenocortical adenomas, even those with 17q allelic losses, had a greater PKA activity ratio, which is an indirect measure of RIα activity (59). These data agreed well with decreased *PRKAR1A* mRNA presence in cancers *versus* adenomas (Fig. 5). Despite their lower total kinase activity, adrenocortical carcinomas retained higher cAMP-induced kinase activity in the presence of PKI, a PKA-specific inhibitor. These data may indicate not only that RIα activity is decreased in adrenocortical cancer independently of the status of the 17q locus (as suggested by the PKA activity ratio) but also that cAMP in these tumors induces non-PKA-dependent alterations in total kinase activity through pathways that may be differentially expressed in cancer *versus* adenomas (60).

The lack of *PRKAR1A* mutations in AC and their presence in adrenocortical adenomas, which is the reverse of the situation in thyroid tumors (29), suggest that diverse tumorigenic processes with regards to the role of PKA exist in endocrine cells. These observations parallel our clinical experience; in >350 patients with CNC and/or PPNAD known to us, there has never been a case of adrenocortical cancer, in contrast to several cases of thyroid carcinomas (61). We speculate that *PRKAR1A* down-regulation through mutations, allelic haploinsuffiency, or epigenetic silencing may participate in the evolution of thyroid cancer toward more undifferentiated forms, as it has been described in thyroid oncogenesis (62) in a linear model and as reported in other tissues (63). In the adrenal cortex, however (and perhaps in the earlier stages of thyroid tumorigenesis), two alternative and not mutually exclusive hypotheses may be proposed: (*a*) *PRKAR1A*'s down-regulation could be a very early step in carcinogenesis and, therefore, quickly bypassed by other pathways more critical for the survival of neoplastic cells; some of these signaling cascades may be cAMP-linked but not necessarily PKA-dependent, as in other tissues (60, 64); and (*b*) when it occurs, especially in the form of *PRKAR1A*-inactivating mutations accompanied by LOH, it may lead to a well-differentiated adenoma with PPNAD-like functional properties that is very unlikely to ever proceed to AC, just like *Lkb1* $-/-$ cells appear to be resistant to at least one form of malignant transformation (64). The reduction of the expression of the PKA-regulated transcription factor cAMP-responsive element binding protein in adrenocortical cancer (13, 14, 65) is further supportive of the relatively decreased role that cAMP-dependent PKA signaling may

[6] C. A. Stratakis and F. Sandrini, unpublished observations.

play in at least the late stages of malignant adrenocortical transformation.

In conclusion, the present study supports a role for *PRKAR1A* in the formation of sporadic adrenocortical adenomas; in some of these tumors, *PRKAR1A* inactivation is associated with CS that shares some of the characteristics of cortisol hypersecretion observed in Carney complex and/or PPNAD.

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REFERENCES

1. Stratakis, C. A., and Chrousos, G. P. Adrenal cancer. Endocrinol. Metab. Clin. North Am., *29:* 15–25, 2000.
2. Varley, J. M., McGown, G., Thorncroft, M., James, L. A., Margison, G. P., Forster, G., Evans, D. G., Harris, M., Kelsey, A. M., and Birch, J. M. Are there low-penetrance *TP53* alleles? Evidence from childhood adrenocortical tumors. Am. J. Hum. Genet., *65:* 995–1006, 1999.
3. Bornstein, S. R., and Stratakis, C. A., and Chrousos, G. P. Adrenocortical tumors: recent advances in basic concepts and clinical management. Ann. Intern. Med., *130:* 759–771, 1999.
4. Gicquel, C., Bertherat, J., Le Bouc, Y., and Bertagna, X. Pathogenesis of adrenocortical incidentalomas and genetic syndromes associated with adrenocortical neoplasms. Endocrinol. Metab. Clin. North Am., *29:* 1–13, 2000.
5. Logie, A., Boulle, N., Gaston, V., Perin, L., Boudou, P., Le Bouc, Y., and Gicquel, C. Autocrine role of IGF-II in proliferation of human adrenocortical carcinoma NCI H295R cell line. J. Mol. Endocrinol., *23:* 23–32, 1999.
6. Wilkin, F., Gagne, N., and Paquette, J. Oligny, L. L., and Deal, C. Pediatric adrenocortical tumors: molecular events leading to insulin-like growth factor II gene overexpression. J. Clin. Endocrinol. Met., *85:* 2048–2056, 2000.
7. Vanttinen, T., Kuulasmaa, T., Liu, J., and Voutilainen, R. Expression of activin/inhibin receptor and binding protein genes and regulation of activin/inhibin peptide secretion in human adrenocortical cells. J. Clin. Endocrinol. Metab., *87:* 4257–4263, 2002.
8. Matzuk, M. M., Finegold, M. J., Mather, J. P., Krummen, L., Lu, H., and Bradley, A. Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. Proc. Natl. Acad. Sci. USA, *91:* 8817–8821, 1994.
9. Kjellman, M., Roshani, L., Teh, B. T., Kallioniemi, O. P., Hoog, A., Gray, S., Farnebo, L. O., Holst, M., Backdahl, M., and Larsson, C. Genotyping of adrenocortical tumors: very frequent deletions of the MEN1 locus in 11q13 and of a 1-centimorgan region in 2p16. J. Clin. Endocrinol. Metab., *84:* 730–735, 1999.
10. Figueiredo, B. C., Stratakis, C. A., Sandrini, R., DeLacerda, L., Pianovsky, M. A., Giatzakis, C., Young, H. M., and Haddad, B. R. Comparative genomic hybridization analysis of adrenocortical tumors of childhood. J. Clin. Endocrinol. Metab., *84:* 1116–1121, 1999.
11. Takahashi, K., Totsune, K., Murakami, O., and Shibahara, S. Expression of urotensin II and urotensin II recptor mRNAs in various human tumor cell lines and secretion of urotensin II-like immunoreactivity by SW-13 adrenocortical carcinoma cells. Peptides, *22:* 175–179, 2001.
12. Martinerie, C., Gicquel, C., Louvel, A., Laurent, M., Schofield, P. N., and Le Bouc, Y. Altered Expression of novH is associated with human adrenocortical tumorigenesis. J. Clin. Endocrinol. Metab., *86:* 3929–3940, 2001.
13. Peri, A., Luciani, P., Conforti, B., Baglioni-Peri, S., Cioppi, F., Crescioli, C., Ferruzzi, P., Gelmini, S., Arnaldi, G., Nesi, G., Serio, M., Mantero, F., and Mannelli, M. Variable expression of the transcription factors cAMP response element-binding protein and inducible cAMP early repressor in the normal adrenal cortex and in adrenocortical adenomas and carcinomas. J. Clin. Endocrinol. Metab., *86:* 5443–5449, 2001.
14. Groussin, L., Massias, J. F., Bertagna, X., and Bertherat, J. Loss of expression of the ubiquitous transcrition factor cAMP response element-binding protein (CREB) and compensatory overexpression of the activator CREMtau in the human adrenocotical cancer cell line H295R. J. Clin. Endocrinol. Metab., *85:* 345–354, 2000.
15. Beuschlein, F., Fassnacht, M., Klink, A., Allolio, B., and Reincke, M. ACTH-receptor expression, regulation and role in adrenocortial tumor formation. Eur. J. Endocrinol., *144:* 199–206, 2001.
16. Reincke, M. Mutations in adrenocortical tumors. Horm. Metab. Res., *30:* 447–455, 1998.
17. Bourdeau, I., and Stratakis, C. A. Cyclic AMP-dependent signaling aberrations in macronodular adrenal disease. Ann. N. Y. Acad. Sci., *968:* 240–255, 2002.
18. Kirschner, L. S. Signaling pathways in adrenocortical cancer. Ann. N. Y. Acad. Sci., *968:* 222–239, 2002.
19. Kirschner, L. S., Carney, J. A., Pack, S. D., Taymans, S. E., Giatzakis, C., Cho, Y. S., Cho-Chung, Y. S., and Stratakis, C. A. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. Nat. Genet., *26:* 89–92, 2000.
20. Kirschner, L. S., Sandrini, F., Monbo, J., Lin, J. P., Carney, J. A., and Stratakis, C. A. Genetic heterogeneity and spectrum of mutations of the *PRKAR1A* gene in patients with the carney complex. Hum. Mol. Genet., *9:* 3037–3046, 2000.
21. Carney, J. A., and Young, W. F. Primary pigmented nodular adrenocortical disease and its associated conditions. Endocrinologist., *2:* 6–21, 1992.
22. Stratakis, C. A. Genetics of Peutz-Jeghers syndrome, Carney complex and other familial lentiginoses. Horm. Res., *54:* 334–343, 2000.
23. Stratakis, C. A. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit (*PRKAR1A*) in patients with the "complex of spotty skin pigmentation, myxomas, endocrine overactivity, and schwannomas" (Carney complex). Ann. N. Y. Acad. Sci., *968:* 3–21, 2002.
24. Cho-Chung, Y. S., and Clair, T. The regulatory subunit of cAMP-dependent protein kinase as a target for chemotherapy of cancer and other cellular dysfunctional-related diseases. Pharmacol. Ther., *60:* 265–288, 1993.
25. van Oirschot, B. A., Stahl, M., Lens, S. M., and Medema, R. H. Protein kinase A regulates expression of p27(kip1) and cyclin D3 to suppress proliferation of leukemic T cell lines. J. Biol. Chem., *276:* 33854–33860, 2001.
26. Sarlis, N. J., Chrousos, G. P., Doppman, J. L., Carney, J. A., and Stratakis, C. A. Primary pigmented nodular adrenocortical disease: reevaluation of a patient with carney complex 27 years after unilateral adrenalectomy. J. Clin. Endocrinol. Metab., *82:* 1274–1278, 1997.
27. Stratakis, C. A., Sarlis, N., Kirschner, L. S., Carney, J. A., Doppman, J. L., Nieman, L. K., Chrousos, G. P., and Papanicolaou, D. A. Paradoxical response to dexamethasone in the diagnosis of primary pigmented nodular adrenocortical disease. Ann. Intern. Med., *131:* 585–591, 1999.
28. Stratakis, C. A., Kirschner, L. S., and Carney, J. A. Clinical and molecular features of the Carney complex: diagnostic criteria and recommendations for patient evaluation. J. Clin. Endocrinol. Metab., *86:* 4041–4046, 2001.
29. Sandrini, F., Matyakhina, L., Sarlis, N. J., Farmakidis, C., Kirschner, L. S., Gimm, O., and Stratakis, C. A. Regulatory subunit type I- of protein kinase A (*PRKAR1A*): a tumor-suppressor gene for sporadic thyroid cancer. Genes Chromosomes Cancer, *35:* 182–192, 2002.
30. Groussin, L., Perlemoine, K., Contesse, V., Tabarin, A., Thieblot, P., Schlienger, J. L., Luton, J. P., Bertagna, X., and Bertherat, J. The ectopic expression of the gastric inhibitory peptide (GIP) receptor is frequent in ACTH-independent macronodular adrenal hyperplasia but rare in benign adrenal adenomas. J. Clin. Endocrinol. Metabol., *87:* 1980–1985, 2002.
31. Luton, J. P., Martinez, M., Coste, J., and Bertherat, J. Outcome in patients with adrenal incidentaloma selected for surgery: an analysis of 88 cases investigated in a single clinical center. Eur. J. Endocrinol., *143:* 111–117, 2000.
32. Gicquel, C., Bertagna, X., Gaston, V., Coste, J., Baudin, E., Bertherat, J., Chapuis, Y., Duclos, J. M., Louvel, A., Plouin, P. F., Luton, J. P., and Le Bouc, Y. Molecular markers and long term recurrences in a large cohort of patients with sporadic adrenocortical tumors. Cancer Res., *61:* 6762–6767, 2001.
33. Kirschner, L. S., and Stratakis, C. A. Large-scale preparation of sequence-ready bacterial artificial chromosome DNA using QIAGEN columns. Biotechniques, *27:* 72–74, 1999.
34. Stratakis, C. A., Taymans, S. E., Daruwala, R., Song, J., and Levine, M. Mapping of the human genes (*SLC23A2* and *SLC23A1*) coding for vitamin C transporters 1 and 2 (SVCT1 and SVCT2) to 5q23 and 20p12, respectively. J. Med. Genet. *37:* E20(1–3), 2000.
35. Stratakis, C. A., Jenkins, R. B., Pras, E., Mitsiadis, C. S., Raff, S. B., Stalboerger, P. G., Tsigos, C., Carney, J. A., and Chrousos, G. P. 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., *81:* 3607–3614, 1996.
36. Groussin, L., Jullian, E., Perlemoine, K., Leheup, B., Luton, J. P., Bertagna, X., and Bertherat, J. Mutations of the *PRKAR1A* gene in Cushing's syndrome due to sporadic primary pigmented nodular adrenocortical disease (PPNAD). J. Clin. Endocrinol. Metab., *87:* 4324–4329, 2002.
37. Fuller, C. E., and Perry, A. Fluorescense in situ hybridization (FISH) in diagnostic and investigative neuropathology. Brain Pathol., *12:* 67–86, 2002.
38. Obermann, E., Junker, K., Stoehr, R., Dietmaier, W., Zaak, D., Schubert, J., Hofstaedter, F., Knuechel, R., and Hartmann, A. Frequent genetic alterations in flat urothelial hyperplasias and concomitant papillary bladder cancer as detected by CGH, LOH, and FISH analyses. J. Pathol., *199:* 50–57, 2003.
39. Groussin, L., Kirschner, L. S., Vincent-Dejean, C., Perlemoine, K., Jullian, E., Delmer, B., Zacharieva, S., Pignatelli, D., Carney, J. A., Luton, J. P., Bertagna, X., Stratakis, C. A., and Bertherat, J. Molecular analysis of the cyclic AMP-dependent protein kinase A (PKA) regulatory subunit 1A (*PRKAR1A*) gene in patients with Carney complex and/or 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:* 1433–1442, 2002.
40. Casey, M., Vaughan, C. J., He, J., Hatcher, C. J., Winter, J. M., Weremowicz, S., Montgomery, K., Kucherlapati, R., Morton, C. C., and Basson, C. T. Mutations in the protein kinase A R1alpha regulatory subunit cause familial cardiac myxomas and Carney complex. J. Clin. Investig., *106:* 31–38, 2000.
41. Beuschlein, F., Reincke, M., Karl, M., Travis, W. D., Jaursch-Hancke, C., Abdelhamid, S., Chrousos, G. P., and Allolio, B. Clonal composition of human adrenocortical neoplasms. Cancer Res., *54:* 4927–4932, 1994.

42. Kjellman, M., Kallioniemi, O. P., Karhu, R., Hoog, A., Farnebo, L. O., Auer, G., Larsson, C., and Backdahl, M. Genetic aberrations in adrenocortical tumors detected using comparative genomic hybridization correlate with tumor size and malignancy. Cancer Res., *56:* 4219–4223, 1996.
43. Houldsworth, J., and Chaganti, R. S. K. Comparative genomic hybridization: an overview. Am. J. Pathol., *145:* 1253–1260, 1994.
44. Zhao, J., Speel, E. J., Muletta-Feurer, S., Rutimann, K., Saremaslani, P., Roth, J., Heitz, P. U., and Komminoth, P. Analysis of genomic alterations in sporadic adrenocortical lesions. Gain of chromosome 17 is an early event in adrenocortical tumorigenesis. Am. J. Pathol., *155:* 1039–1045, 1999.
45. Barghorn, A., Komminoth, P., Bachmann, D., Rutimann, K., Saremaslani, P., Muletta-Feurer, S., Perren, A., Roth, J., Heitz, P. U., and Speel, E. J. Deletion at 3p25.3-p23 is frequently encountered in endocrine pancreatic tumours and is associated with metastatic progression. J. Pathol., *194:* 451–458, 2001.
46. Zhou, X. P., Gimm, O., Hampel, H., Niemann, T., Walker, M. J., and Eng, C. Epigenetic PTEN silencing in malignant melanomas without PTEN mutation. Am. J. Pathol., *157:* 1123–1128, 2000.
47. Zedenius, J., Wallin, G., Svensson, A., Bovee, J., Hoog, A., Backdahl, M., and Larsson, C. Deletions of the long arm of chromosome 10 in progression of follicular thyroid tumors. Hum. Genet., *97:* 299–303, 1996.
48. Gimm, O., Chi, H., Dahia, P. L., Perren, A., Hinze, R., Komminoth, P., Dralle, H., Reynolds, P. R., and Eng, C. Somatic mutation and germline variants of *MINPP1*, a phosphatase gene located in proximity to *PTEN* on 10q23.3, in follicular thyroid carcinomas. J. Clin. Endocrinol. Metab., *86:* 1801–1805, 2001.
49. Sandrini, F., Bertherat, J., Bei, T., Lorang, D., Libutti, S. K., Kirschner, L. S., Carney, and J. A., and Stratakis, C. A. Effectors of tumorigenicity in Primary Pigmented Nodular Adrenocortical Disease (PPNAD): Protein kinase-A (PKA) subunit expression and activity, laser-capture microdissection (LCM) and CREB phosphorylation. (Abstract OR-36.2), pp. 113, Proceedings of the 84th Annual Endocrine Society Meeting, San Francisco, June 2002.
50. Bertherat, J. Protein kinase A in Carney complex: a new example of cAMP pathway alteration in endocrine tumors. Eur. J. Endocrinol., *144:* 209–211, 2001.
51. Otten, A. D., Parenteau, L. A., Doskeland, S. O., and McKnight, G. S. Hormonal activation of gene transcription in ras-transformed NIH3T3 cells over-expressing RIIα and RIIβ subunits of the cAMP-dependent protein kinase. J. Biol. Chem., *266:* 23074–23082, 1991.
52. McKnight, G. S., Cummings, D. E., Amieux, P., Sikorski, A. M., Brandon, E. P., Planas, J. V., Motamed, K., and Idzerda, R. L. Cyclic AMP, PKA, and the regulation of adiposity. Rec. Prog. Horm. Res., *53:* 139–161, 1998.
53. Garrel, G., Delahaye, R., Hemmings, B. A., and Counis, R. Modulation of regulatory and catalytic subunit levels of cAMP-dependent protein kinase A in anterior pituitary cells in response to direct activation of protein kinases A and C or after GnRH stimulation. Neuroendocrinology, *62:* 514–522, 1995.
54. Lange-Carter, C. A., and Malkinson, A. M. Differential regaulation of stability of cAMP-dependent protein kinase messenger RNA in normal *versus* neoplastic mouse lung epithelial cells. Cancer Res., *51:* 6699–6703, 1991.
55. Weber, W., and Hiltz, H. cAMP-dependent proetin kinases I and II: divergent turnover of subunits. Biochemistry, *25:* 5661–5667, 1986.
56. Scott, J. D. Cyclic nucleotide-dependent protein kinases. Pharmacol. Ther., *50:* 123–145, 1991.
57. Cummings, D. E., Brandon, E. P., Planas, J. V., Motamed, K., Idzerda, R. L., and McKnight, G. S. Genetically lean mice result from targeted disruption of the RIIb subunit of protein kinase A. Nature (Lond.), *382:* 622–626, 1996.
58. Amieux, P. S. Cummings, D. E., Motamed, K., Brandon, E. P., Wailes, L. A., Le, K., Idzerda, R. L., and McKnight, G. S. Compensatory regulation of RIα protein levels in protein kinase A mutant mice. J. Biol. Chem., *272:* 3993–3998, 1997.
59. Cho, Y. S., Park, Y. G., Lee, Y. N., Kim, M. K., Bates, S., Tan, L., and Cho-Chung, Y. S. Extracellular protein kinase A as a cancer biomarker: its expression by tumor cells and reversal by a myristate-lacking Cα and RIIβ subunit overexpression. Proc. Natl. Acad. Sci. USA, *97:* 837–840, 2000.
60. Tsygankova, O. M., Kupperman, E., Wen, W., and Meinkoth, J. L. Cyclic AMP activates Ras. Oncogene, *19:* 3609–3615, 2000.
61. Stratakis, C. A., Courcoutsakis, N., Abati, A., Filie, A., Doppman, J. L., Carney, J. A., and Shawker, T. Thyroid gland abnormalities in patients with the syndrome of spotty skin pigmentation, myxomas, endocrine overactivity, and schwannomas (Carney complex). J. Clin. Endocrinol. Metab., *82:* 2037–2043, 1998.
62. Eng, C. Familial papillary thyroid cancer–many syndromes, too many genes? J. Clin. Endocrinol. Metab., *85:* 1755–1757, 2000.
63. Kinzler, K. W., and Vogelstein, B. Lessons from hereditary colorectal cancer. Cell, *87:* 159–170, 1996.
64. Bardeesy, N., Sinha, M., Hezel, A. F., Signoretti, S., Hathaway, N. A., Sharpless, N. E., Loda, M., Carrasco, D. R., and DePinho, R. A. Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. Nature (Lond.), *419:* 162–167, 2002.
65. Rosenberg, D., Groussin, L., Julian, E., Perlemoine, K., Medjane, S., Louvel, A., Bertagna, X., and Bertherat, J. The transcription factor CREB (cAMP Responsive Element Binding Protein) is decreased in adrenocortical malignant tumors and during adrenal development. (Abstract P3-495), pp. 607, Proceedings of the 84th Annual Endocrine Society Meeting, San Francisco, June 2002.