0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(3):1332–1339

doi: 10.1210/jc.2003-031065

Expression of the Novel Adrenocorticotropin-Responsive Gene Selective Alzheimer's Disease Indicator-1 in the Normal Adrenal Cortex and in Adrenocortical Adenomas and Carcinomas

PAOLA LUCIANI, PIETRO FERRUZZI, GIORGIO ARNALDI, CLARA CRESCIOLI, SUSANNA BENVENUTI, GABRIELLA NESI, ANDREA VALERI, ISABELL GREEVE, MARIO SERIO, MASSIMO MANNELLI, AND ALESSANDRO PERI

Endocrine Unit (P.L., P.F., C.C., S.B., M.S., M.M., A.P.), Department of Clinical Physiopathology and Department of Human Pathology and Oncology (G.N.), University of Florence, 50139 Florence, Italy; Division of Endocrinology, Department of Internal Medicine (G.A.), University of Ancona, 60131 Ancona, Italy; General Surgery Unit (A.V.), Ospedale Careggi, 50134 Florence, Italy; and Department of Neurology (I.G.), Inselspital-University Hospital, 3010 Bern, Switzerland

Selective Alzheimer's disease indicator-1 (seladin-1) is a novel gene with antiapoptotic activity that is down-regulated in vulnerable brain regions in Alzheimer's disease. This gene encodes 3-β-hydroxysterol Δ-24-reductase (DHCR24), which converts desmosterol into cholesterol. In the adrenal cortex, increased expression of seladin-1/DHCR24, which appears to be modulated by ACTH, has been recently reported in cortisol-secreting adenomas, compared with the adjacent atrophic tissue. In our study, we measured the expression level of seladin-1/DHCR24 in cortisol- (n = 18) and aldosterone-secreting (n = 16) adrenocortical adenomas, in carcinomas (n = 17), and in normal adrenal glands (n = 8) by quantitative real-time RT-PCR. The amount of seladin-1/DHCR24 mRNA was significantly reduced in carcinomas (total RNA, 2.5 ± 0.8 pg/μg) compared with the other groups ($P < 0.01$). Western blot analysis confirmed the mRNA results. Similarly, in adrenal malignancies, significantly reduced levels of expression of the ACTH receptor gene were found. In the adrenal cancer cell line H295R and in primary cultures from adrenocortical cells, ACTH (1 nM) and forskolin (10 μM) effectively increased seladin-1/DHCR24 expression, confirming that seladin-1/DHCR24 is modulated by the ACTH/cAMP-driven pathway. In summary, this is the first demonstration that seladin-1/DHCR24 expression is reduced in adrenal cancer, suggesting that it might be viewed as a new potential marker of adrenal malignancies. (*J Clin Endocrinol Metab* 89: 1332–1339, 2004)

THE MOLECULAR ALTERATIONS characterizing adrenocortical tumors have begun to be unraveled only in the last decade and, therefore, are still only partially known. ACTH regulates cell differentiation, steroid synthesis, and, although to a lesser extent, cell proliferation (1). Activating mutations of the ACTH receptor gene have not been found in adrenocortical tumors to date (reviewed in Ref. 2). On the other hand, loss of heterozygosity of the ACTH receptor gene and reduced levels of the corresponding transcript have been observed in adrenal cancer (3), and, in addition, we have recently described loss of expression of the cAMP-dependent transcription factors cAMP response element binding protein and inducible cAMP early repressor in a subset of adrenocortical carcinomas (4). The activation of the proto-oncogene *K-ras*, p53 mutations, and loss of heterozygosity at 11q13, but no mutation in the *MEN 1* gene, allelic loss at 11p15, and overexpression of IGF-II and IGF-binding protein-2 or epidermal growth factor receptor have been observed with variable frequency in adrenal cancer (reviewed in Ref. 2). However, the relevance of these findings to adrenal tumorigenesis remains controversial.

Recently, by using a suppression PCR-based cDNA subtractive hybridization technique to isolate genes differentially expressed in different cDNA samples, Sarkar *et al.* (5) reported overexpression of a newly identified gene, named selective Alzheimer's disease indicator-1 (seladin-1), in benign cortisol-producing adrenocortical adenomas compared with the atrophic adjacent gland. However, a semiquantitative analysis was performed. This gene is the human homolog of the Diminuto/Dwarf1 gene, described in plants (*i.e. Arabidopsis thaliana*) and in *Caenorhabditis elegans* (6). In plants, Diminuto/Dwarf1 is required for the synthesis of brassinosteroids, which are plant sterols essential for normal growth and development (7, 8). In humans, the seladin-1 gene has been identified and found to be down-regulated in brain regions affected by Alzheimer's disease (9). Seladin-1, which is mainly located in the endoplasmic reticulum, confers resistance to neuronal cells from β-amyloid toxicity and from oxidative stress. In addition, it inhibits caspase 3 activity, a key mediator of apoptosis, and protects from apoptotic death (9). A subsequent study identified seladin-1 gene as the gene encoding 3-β-hydroxysterol Δ-24-reductase (DHCR24) (10). This enzyme catalyzes the reduction of the Δ 24 double bond in desmosterol to produce

Abbreviations: DHCR24, 3-β-Hydroxysterol Δ-24-reductase; seladin-1, selective Alzheimer's disease indicator-1.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

cholesterol. Mutations of this gene have been found in desmosterolosis, a rare, severe, multiple-congenital anomaly syndrome, which involves developmental and growth retardation.

According to the experimental data on neuronal cells, in the adrenal cortex as well, the expression of seladin-1/DHCR24, which appeared to be up-regulated by ACTH in rats, has been related to reduced apoptosis (5). The authors hypothesized that seladin-1/DHCR24 might be related to the molecular events of adrenocortical tumorigenesis.

In the present study, we focused on the expression of seladin-1/DHCR24 in a rather large series of adrenocortical adenomas and carcinomas and normal adrenal glands by using a quantitative method (real-time RT-PCR) for the measurement of mRNA levels and Western blotting. In addition, we determined the effect of ACTH on seladin-1/DHCR24 expression in cultured human adrenocortical cells. Finally, in our series of adrenal tissues, we determined the levels of the ACTH receptor transcript.

Patients and Methods

Patients

Fifty-one patients who were undergoing surgery for an adrenocortical mass (aldosterone-secreting adenoma, n = 16, patients no. 1–16; cortisol-secreting adenoma, n = 18, patients no. 17–34; and carcinoma, n = 17, patients no. 35–51) were included in the study after informed consent was obtained. Eight additional patients, who were undergoing nephrectomy for renal cancer, were included in the study. Tissue specimens from the adrenal cortex that were obtained at surgery were immediately frozen in liquid nitrogen and stored at −80 C until RNA and protein extraction. The clinical data of the patients are reported in Table 1.

Primary cell cultures

Adrenocortical fragments obtained at surgery were also processed for cell preparation. Primary cell cultures from normal adrenal zona fasciculata (n = 2), cortisol-secreting adenomas (n = 3), and adrenal carcinoma (n = 1) were established within 1 h from surgery, as described by Munari-Silem *et al.* (11), with some modifications. Briefly, normal adrenals were freed of fat, decapsulated, and the subcapsular zone and the medulla were removed using a scalpel and discarded. Adenoma and carcinoma zones, macroscopically distinguishable from normal areas, were decapsulated as necessary and cut and picked up from the inner part to ensure tissue-type specificity. Thereafter, tissues were minced and incubated for 20 min at 37 C in PBS containing 2 mg/ml collagenase. To facilitate dispersion, tissues were minced with a Pasteur pipette with a fine heat-polished tip, and the cell suspension was then filtered through a cell strainer (80 μm mesh; Sigma Chemical Co., St. Louis, MO) and centrifuged for 10 min at 1400 rpm. The pellet was resuspended in a culture medium consisting of a 1:1 (vol/vol) mixture DMEM/F-12 with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and enriched with a mixture of insulin, transferrin, and selenium. Isolated cells were plated onto 35-mm diameter culture dishes at a density of 50–70 $\times$ 10^3 cells/dish and cultured at 37 C in 95% air-5% CO_2 in a fully humidified environment. Primary cultures were used within 3–4 d. The precise number of cells used in each experimental protocol was determined at the end of the experiments using a hemocytometer. The mesodermal origin of the cells was assessed by the positive staining observed by using a mouse antihuman monoclonal antibody against vimentin (CLONE V9) (data not shown).

For metabolic characterization of zona fasciculata cells, cortisol secretion was tested along with 17-hydroxyprogesterone and aldosterone secretion, after 24 h of growth in the growth medium, in all cell preparations. Cortisol was, by far, the main secretory product of zona fasciculata cells, whereas a lower amount of 17-hydroxyprogesterone and virtually no aldosterone were detected (data not shown). The hormonal secretory profile was also assessed in primary cell cultures from cortisol-secreting adenomas (cortisol secretion was confirmed) and from the adrenocortical carcinoma (no secretion).

Quantitative real-time RT-PCR for seladin-1/DHCR24 transcript

The measurement of seladin-1 transcript was performed by quantitative real-time RT-PCR, based on TaqMan technologies, on total RNAs extracted from all tissue samples obtained at surgery and from cultured cells, according to the method described by Chomczynski and Sacchi (12). With regard to tumoral samples, multiple sampling was performed at the time of surgery well inside the tumor to avoid normal tissue contamination, and small tissue fragments were sent to the pathologist for histological assessment. The presence of necrosis was always checked, and the RNA was extracted from tissue samples in which the necrosis was limited to a very few focal areas. RNA concentration was determined spectrophotometrically by absorption at 260 nm, and the quality/integrity was assessed by the 260:280 absorption ratio and by gel electrophoresis of total RNAs. The real-time RT-PCR was performed using primers and probe selected by the proprietary software Primer Express (Applied Biosystems Inc., Foster City, CA). The sequences of seladin-1/DHCR24 primers, spanning 88 bp were 5′-ATCGCAGCTTTGTGCGATG-3′ (sense, exon 4, 5′-end at position 686) and 5′-CACCAGGAAACCCAGCGT-3′ (antisense, exon 5, 5′-end at position 774). The sequence of the probe, labeled with 6-carboxy-fluorescein, which hybridized to the exon 4–5 junction region, was 5′-TCCGTCCGAAAACTCAGACCTGTTCTATGC-3′ (5′-end at position 708). Total RNA (400 ng) was reverse transcribed in 80 μl of final volume using the TaqMan Universal Master Mix (Applied Biosystems), following the manufacturer's instructions. The profile of the one-step, reverse-transcription reaction was 10 min at 25 C, 30 min at 48 C, and 5 min at 95 C. The cDNAs were then subjected to PCR using the following conditions: 2 min at 50 C and 10 min at 95 C, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min in the ABI Prism 7700 Sequence Detector (Applied Biosystems). A calibration curve was generated using a single-stranded sense oligodeoxynucleotide spanning the sequence included between the primers, as described by Bustin (13).

All the reactions were run in triplicates in the presence of no template controls. Because normalization to rRNA or to glyceraldehyde-3-phosphate dehydrogenase as well as to other housekeeping genes has been clearly shown to not be accurate (13, 14), the results were expressed as pg seladin-1/DHCR24 mRNA/μg total RNA.

Ki67 and bcl-2 assessment

For the immunohistochemical assessment of Ki67 and bcl-2, a representative section for each lesion was selected. All sections were dewaxed in Bio-Clear (Bio-Optica, Milan, Italy) and hydrated with grade ethanol concentration until distilled water. Antigen retrieval was routinely performed by microwave pretreatment (Microwave MicroMed T/T Mega, Milestone, Bergamo, Italy) in citrate buffer (10 mM, pH 6.0) for 30 min. As primary antibodies, the following two commercial mouse monoclonal antibodies were used: anti-bcl-2, clone bcl-2/100/D5 (Ventana Medical Systems, Tucson, AZ) raised against bcl-2 oncoprotein, and antihuman Ki67 antigen, clone MIB-1 (Dako Cytomation, Glostrup, Denmark) raised against the nuclear protein Ki67. All tissue sections were then placed in the Ventana Nexes automated stainer using the iVIEW DAB Detection Kit (Ventana Medical Systems) as revelation system. After the staining run was complete, the tissue sections were removed from the stainer, counterstained with hematoxylin, dehydrated, and mounted with Permount. Formalin-fixed, paraffin-embedded sections of reactive tonsils were used as positive control for both antibodies. For negative controls, omission of the primary antibodies was performed. A positive MIB-1 phenotype was considered when more than 5% of tumor cells showed nuclear immunoreactivity, whereas bcl-2 staining was considered positive when more than 10% of cells showed cytoplasmatic immunoreactivity.

Protein extraction

Tissue samples for protein extraction were available only in a subset of cases because the same samples had been extensively used for other studies. Frozen tissues were ground and kept in lysis buffer [20 mM

TABLE 1. Clinical features of the patients affected by adrenocortical aldosterone- (patient nos. 1–16) and cortisol- (patient nos. 17–34) secreting adenomas and carcinomas (patient nos. 35–51)

Patient no.	Age (yr)	Sex	Stage at surgery	Postsurgical outcome	Tumor size (cm)	Tumor secretion
1	59	M		Cured	1.5	MC
2	60	M		Cured	2.3	MC
3	41	F		Cured	3.4	MC
4	45	F		Cured	2.5	MC
5	38	M		Cured	2.1	MC
6	46	F		Cured	1.7	MC
7	48	F		Cured	2.5	MC
8	36	F		Cured	2	MC
9	52	M		Cured	1.9	MC
10	58	M		Cured	1	MC
11	63	F		Cured	3	MC
12	24	F		Cured	3.5	MC
13	53	M		Cured	4	MC
14	48	F		Cured	2	MC
15	61	M		Cured	1.5	MC
16	50	M		Cured	2.5	MC
17	23	F		Cured	8	C
18	43	F		Cured	3	C
19	74	F		Cured	4	C
20	50	F		Cured	3	C
21	31	F		Cured	3.5	C
22	48	F		Cured	3	C
23	67	M		Cured	4	C
24	72	M		Cured	4	C
25	54	F		Cured	3	C
26	42	M		Cured	2	C
27	58	F		Cured	7	C
28	46	F		Cured	3	C
29	59	M		Cured	6	C
30	60	M		Cured	2	C
31	62	F		Cured	10	C
32	45	F		Cured	4	C
33	37	F		Cured	3	C
34	55	M		Cured	2	C
35	47	F	II	Unknown	12	C
36	56	M	I	Died, 2 yr[a]	14	None
37	69	M	II	Died, 5 yr	13	None
38	21	F	IV	Died, 1 yr	12	C
39	44	M	IV	Died, 1 yr	15	C
40	54	F	II	Recurrence	13	None
41	46	F	III	Remission, 8 yr	8	None
42	17	F	III	Died, 6 months	13	C
43	58	F	II	Recurrence	11	C
44	69	F	III	Died, 4 yr	6	MC + A
45	26	F	IV	Died, 2 yr	9	C
46	51	F	III	Recurrence, mit	8	None
47	28	F	III	Died, 3 yr	22	A
48	33	F	II	Recurrence, 4 yr, mit	5	A
49	71	F	IV	Died, 2 yr	8	None
50	35	F	II	Metastasis, 4 yr, mit	5	C
51	35	F	III	Remission, 2 yr, mit	12	A

M, Male; F, female; mit, mitotane treatment; MC, mineralcorticoids; C, cortisol; A, androgens.
[a] Duration of follow-up, when available.

Tris-HCl, 150 mM NaCl, 0.2 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 × phosphatase inhibitor cocktail 1 and 1 × phosphatase inhibitor cocktail 2 (Sigma)] for 2 h at 4 C. Thereafter, protein concentration was determined.

Western blot analysis for seladin-1/DHCR24

Proteins (30 μg) were diluted in equal volume of reducing 2 × Laemmli's sample buffer (62.5 mM Tris, pH 6.8; containing 10% glycerol, 2% sodium dodecyl sulfate, 2.5% pyronin, and 200 mM dithiothreitol), incubated at 95 C for 5 min, and loaded onto 10% polyacrylamide-bisacrylamide gel. After separation in SDS-PAGE, proteins were transferred onto nitrocellulose (Sigma) and stained with Ponceau to verify equal protein loading. The nitrocellulose was blocked in 5% skim milk powder for 1 h in TTBS solution (Tris-buffered saline containing 0.1% Tween 20, pH 7.4), washed, and then immunostained with a rabbit polyclonal anti-Seladin-1/DHCR24 antibody (1:2000), specific for the 60-kDa seladin-1 protein, kindly provided by Dr. Isabell Greeve (University of Bern, Bern, Switzerland), followed by a peroxidase-conjugated secondary antirabbit IgG antibody (1:2000; New England Biolabs, Beverly, MA). Antigen-antibody complexes were detected by incubation with LumiGLO chemiluminescent reagent and peroxide (New England Biolabs) and exposure to autoradiography films. Quantification was obtained by densitometric scanning.

In vitro studies

Primary cell cultures and H295R cells (human adrenocortical carcinoma cell line; American Type Culture Collection, Manassas, VA) were seeded in six-well plates. After 3–4 d of culture, the growth medium was removed, and the cells were accurately washed in PBS and incubated in phenol red- and serum-free medium containing 0.1% BSA. After 24 h, ACTH-(1–24) (1 nM) or forskolin (10 μM) was added to the cultures. After 2, 8, 12, 24, 48, or 72 h harvesting, cells were trypsinized and counted using a hemocytometer. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as basal controls. The experiments were repeated three times. Total RNA was extracted as described previously. Cortisol secretion in primary cultures (basal and after ACTH treatment) was evaluated by measuring cortisol in the medium using an electrochemiluminescent assay (Roche Diagnostics, Monza, Italy).

ACTH receptor expression

The gene expression of the ACTH receptor was evaluated by quantitative/competitive RT-PCR, as described previously (15). The measurement was performed in total RNA extracted from normal adrenal glands (n = 8), from aldosterone- (n = 16) and cortisol-secreting (n = 18) adenomas, from those adrenal carcinomas in which RNA was available (n = 8), and from H295R cells. Briefly, the following primers were used: 5′-ACTGTCCTCGTGTGGTTTTG-3′ (sense) and 5′-AGATGAAGACCCCGAGCAG-3′ (antisense). A nonhomologous competitor was constructed using a 300-bp core sequence from pBluescript (Stratagene, La Jolla, CA) with ACTH receptor primer ends, prepared by PCR. There was a 66-bp difference between the length of the competitor and the normal ACTH receptor transcript. After RNA synthesis, five increasing amounts of competitor were mixed with fixed amounts of tumoral RNA after RNase-free DNase digestion. The bands corresponding to the competitor and the transcript products were resolved by gel electrophoresis. The densitometric ratios were determined and were then plotted against the amount of competitor RNA added to each RT-PCR reaction. The amount of ACTH receptor mRNA (pg/μg total RNA) was extrapolated considering the value 1 of the competitor to target ratio as the point in which the quantity of competitor is equal to that of the target (tumoral ACTH receptor).

Statistical analysis

Data were analyzed using the Student's *t* test and expressed as mean ± SE. Differences were considered as statistically significant at the 0.05 level.

Results

Quantitative determination of seladin-1/DHCR24 mRNA

The quantitative expression of seladin-1/DHCR24 mRNA was determined from total RNA extracted from the excised adrenal glands by real-time RT-PCR based on TaqMan technologies (see *Patients and Methods*). The results are shown in Fig. 1 and indicate that the levels of seladin-1/DHCR24 mRNA did not significantly differ between the normal adrenal cortex (mean ± SE, 8.8 ± 1.9 pg/μg total RNA) and aldosterone- (10.8 ± 2.2 pg/μg total RNA) or cortisol-secreting (9.7 ± 3.6 pg/μg total RNA) adenomas. Conversely, the levels of seladin-1/DHCR24 mRNA were significantly lower in adrenocortical carcinomas (mean ± SE, 2.5 ± 0.8 pg/μg total RNA) compared with all the other groups ($P < 0.01$). No significant difference was observed between secreting (n = 11) and nonsecreting (n = 6) carcinomas. There was no correlation between the levels of seladin-1/DHCR24 expression and the size of the carcinomas. Conversely, significantly lower levels of seladin-1/DHCR24 mRNA were detected in stage III–IV compared with stage I–II tumors (mean ± SE, 1.1 ± 0.3 *vs.* 4.5 ± 1.6 pg/μg total RNA; $P < 0.05$).

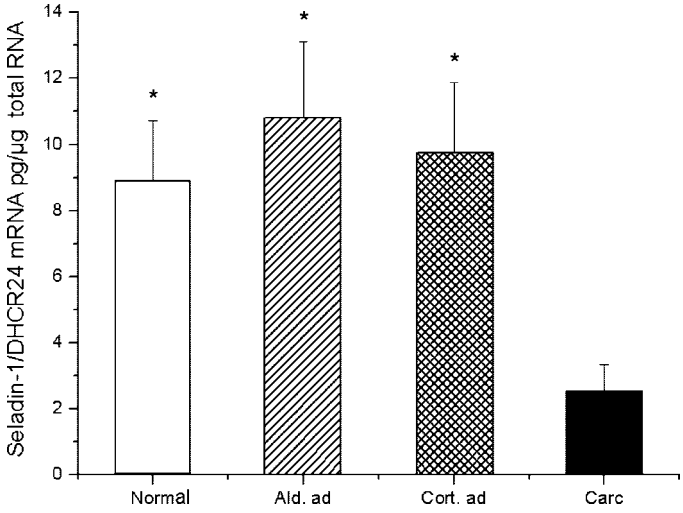

FIG. 1. Seladin-1/DHCR24 mRNA levels in normal adrenal glands, aldosterone- (Ald. ad) and cortisol-secreting (Cort. ad) adenomas, and adrenal carcinomas (Carc), as assessed by real-time RT-PCR. *, $P < 0.01$ *vs.* carcinomas.

Tumoral tissues were further characterized. Cell proliferation status was assessed by determining the Ki67 index. No significant difference in the percentage of nuclei that stained positively was observed between cortisol- and aldosterone-secreting adenomas (mean ± SE, 1.35 ± 0.35% *vs.* 1.15 ± 0.25%), whereas Ki67 positivity was significantly higher in adrenal carcinomas (mean ± SE, 8.63 ± 2.14%; $P < 0.05$ *vs.* cortisol-secreting adenomas and $P < 0.01$ *vs.* aldosterone-secreting adenomas). Immunostaining for bcl-2, a regulator of apoptosis, was also assessed. No benign or malignant tumor showed positivity, according to a cutoff of 10% of immunoreactive cells.

Detection of seladin-1/DHCR24 protein

The detection of seladin-1/DHCR24 protein was performed by Western blot analysis in those cases in which there was enough material available (see *Patients and Methods*) (normal adrenal cortex, n = 7; aldosterone-secreting adenomas, n = 7; cortisol-secreting adenomas, n = 8; and adrenal carcinomas, n = 5). The results, shown in Fig. 2, indicate that a seladin-1/DHCR24-specific signal of 60 kDa was detected in all cases. In agreement with the mRNA data, densitometric analysis of the Western blot signals revealed that, although sporadic individual variations were observed, the mean amount of seladin-1/DHCR24 protein was significantly lower in adrenal carcinomas (mean ± SE, 75.4 ± 10.7 arbitrary units) than in the normal adrenal cortex (103.2 ± 3.8 arbitrary units, $P < 0.05$) and in aldosterone- and cortisol-secreting adenomas (221.2 ± 32.2 arbitrary units, $P < 0.01$ and 115.6 ± 6.9 arbitrary units, $P < 0.01$, respectively; Fig. 3). Furthermore, the amount of seladin-1/DHCR24 in aldosterone-secreting adenomas, in which a slight prevalence of the transcript had been detected by real-time RT-PCR experiments, was significantly higher than in all the other groups ($P < 0.01$).

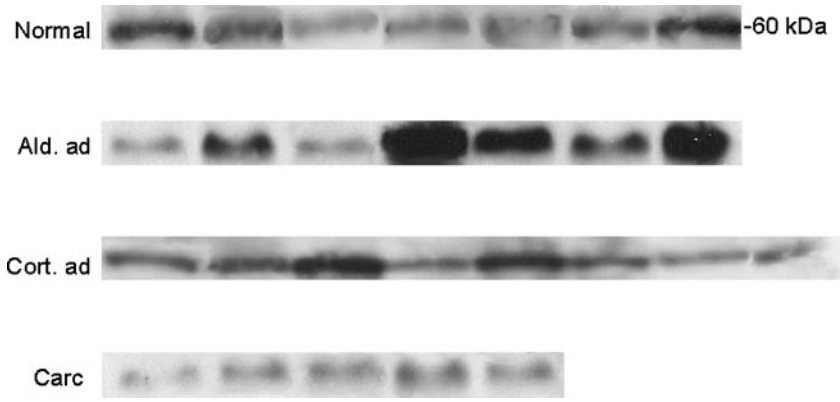

FIG. 2. Western blot analysis of seladin-1/DHCR24 protein in normal adrenal glands, aldosterone- (Ald. ad) and cortisol-secreting (Cort. ad) adenomas, and adrenal carcinomas (Carc).

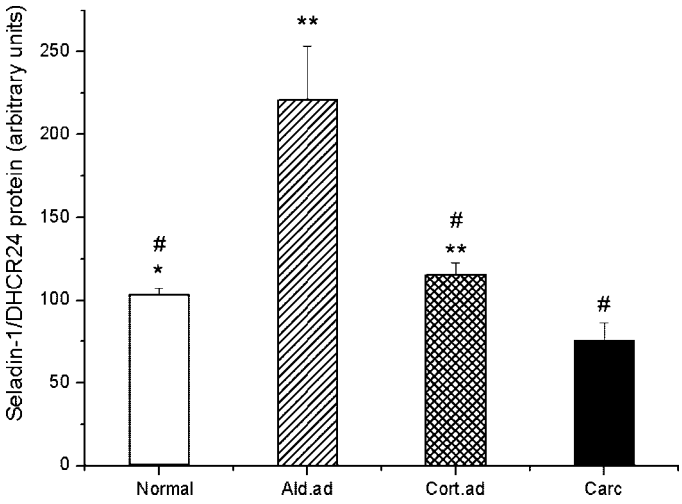

FIG. 3. Densitometric analysis of seladin-1/DHCR24 protein levels in normal adrenal glands, aldosterone- (Ald. ad) and cortisol-secreting (Cort. ad) adenomas, and adrenal carcinomas (Carc). *, $P < 0.05$; **, $P < 0.01$ *vs.* carcinomas; and #, $P < 0.01$ *vs.* aldosterone-secreting adenomas.

Effect of ACTH and forskolin on seladin-1/DHCR24 expression

To determine whether the expression of seladin-1/DHCR24 is modulated by the ACTH/cAMP pathway, *in vitro* studies using the human adrenocortical cancer cell line H295R as well as primary cultures from normal adrenal zona fasciculata (n = 2), cortisol-secreting adenomas (n = 3), and adrenal nonsecreting carcinoma (n = 1) cells were performed. In H295R cells, ACTH (1 nM) determined an increase of seladin-1/DHCR24 mRNA levels, which was observed starting from a 12-h treatment. Prolonged ACTH treatment (24, 48, and 72 h) also resulted in increased amounts of seladin-1/DHCR24 mRNA compared with untreated cells. The results of a representative experiment are shown in Fig. 4A. According to Sarkar *et al.* (5), 10 μM of the adenylate cyclase activator forskolin (12, 48 and 72 h-treatment) also increased the expression levels of seladin-1/DHCR24, substantiating the hypothesis that seladin-1 expression is modulated via the cAMP-signaling pathway. In agreement with the results obtained in H295R cells, a 12-h treatment with 1 nM ACTH in primary cell cultures (normal adrenal zona fasciculata, cortisol-secreting adenomas, and adrenal carcinoma) increased the amount of seladin-1/DHCR24 mRNA in all cases (Fig. 4B and Table 2). Cortisol secretion was also

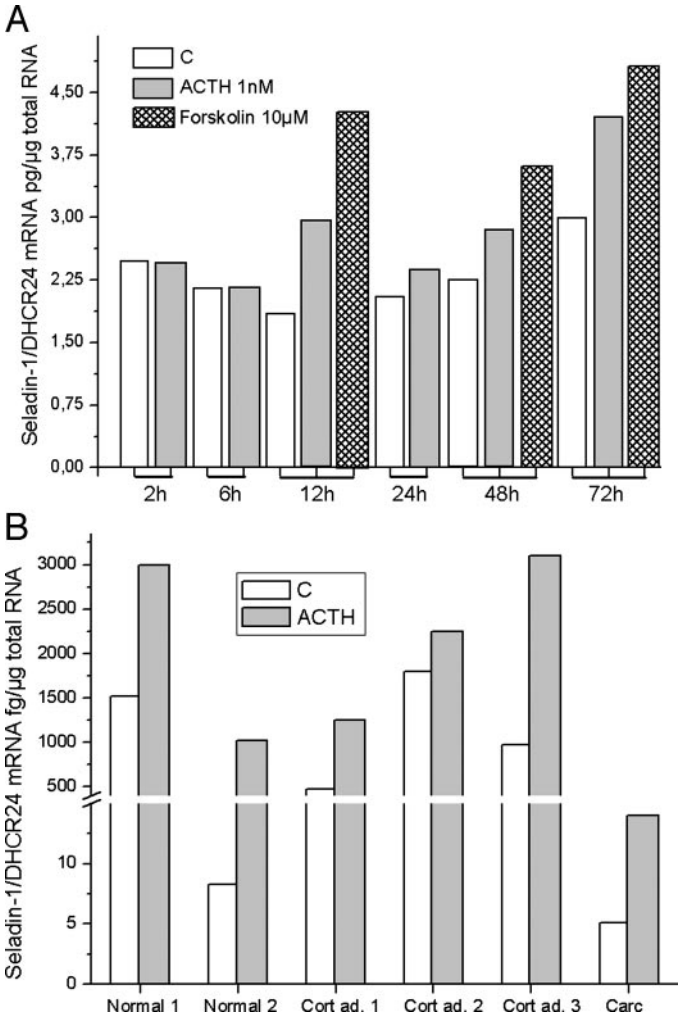

FIG. 4. A, Effect of ACTH (2, 6, 12, 24, 48, and 72 h) or forskolin (12, 48, and 72 h) treatment on seladin-1/DHCR24 mRNA levels in H295R cells compared with untreated cells. B, Effect of a 12-h treatment with ACTH on the levels of seladin-1/DHCR24 mRNA in primary cell cultures [normal adrenal zona fasciculata, n = 2; cortisol-secreting adenomas (Cort ad.), n = 3; and adrenal carcinoma (Carc)]. C, Control untreated cells.

evaluated in basal conditions and after ACTH treatment. In primary cultures from normal adrenal cortex (n = 2) and from cortisol-secreting adenomas (n = 3), there was an increase in the amount of cortisol in the medium after ACTH (Table 2). No cortisol was detected in the medium of cells from the adrenal carcinoma, both in basal conditions and after ACTH treatment.

ACTH receptor expression

Reduced levels of expression of the ACTH receptor in adrenal cancer have been described previously (3, 4). Because seladin-1/DHCR24 expression appears to be regulated by ACTH via the cAMP pathway and because reduced levels of seladin-1/DHCR24 expression were found in adrenal carcinomas, in our series of adrenocortical tissues, the amount of ACTH receptor mRNA was also assessed by quantitative RT-PCR. The results indicated that the levels of ACTH receptor mRNA in normal adrenal cortex and in cortisol-secreting adenomas were not significantly different (mean ± SE, 23.4 ± 3.7 pg/μg total RNA and 30.9 ± 2.7 pg/μg total RNA, respectively; Fig. 5). Conversely, significantly higher expression levels were found in aldosterone-secreting adenomas (40.5 ± 4.1 pg/μg total RNA; $P < 0.01$ *vs.* normal adrenal glands and carcinomas and $P < 0.05$ *vs.* cortisol-secreting adenomas), in agreement with a previous report (15). With regard to adrenal carcinomas, significantly lower amounts of ACTH receptor mRNA were detected compared with all the other groups (9.5 ± 4.9 pg/μg total RNA; $P < 0.01$ *vs.* aldosterone- and cortisol-secreting adenomas and $P < 0.05$ *vs.* normal adrenal glands). The amount of ACTH receptor mRNA was also assessed in H295R cells and was found to be comparable to the amount detected in adrenal carcinomas (11.5 pg/μg total RNA).

TABLE 2. Amount of seladin-1/DHCR24 mRNA levels and cortisol production in primary cultures from normal adrenal cortex, cortisol-secreting adenomas, and carcinoma cells

	Seladin-1 mRNA (fg/μg total RNA)		Cortisol production (nmol/10^6 cells)	
	C	ACTH	C	ACTH
Normal 1	1520	3000	3	10.4
Normal 2	8.25	1020	1.43	3.02
Cort. ad. 1	472	1250	2.53	14.6
Cort. ad. 2	1800	2250	8.7	15.14
Cort. ad. 3	975	3100	10.64	20
Carc.	5.1	14	ND	ND

C, Control untreated cells; ACTH, ACTH-treated cells (1 nM); ND, not detectable; Cort. ad., cortisol-secreting adenomas; Carc., carcinoma.

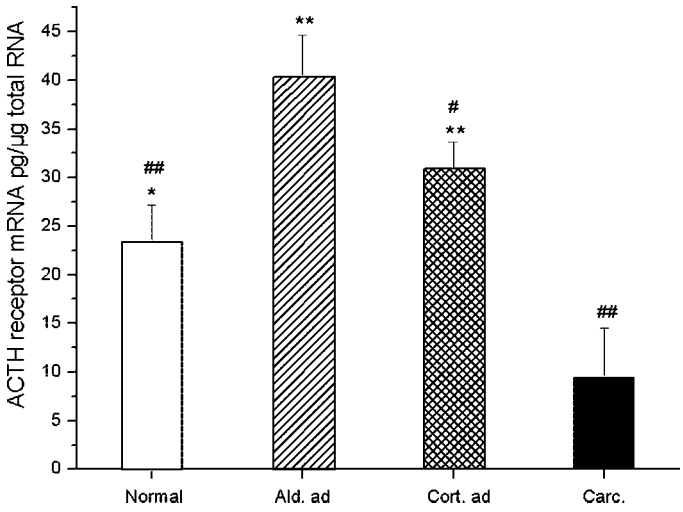

FIG. 5. Amount of ACTH receptor expression in normal adrenal glands, aldosterone- (Ald. ad) and cortisol-secreting (Cort. ad) secreting adenomas, and adrenal carcinomas (Carc), as assessed by competitive RT-PCR. *, $P < 0.05$; **, $P < 0.01$ *vs.* carcinomas; #, $P < 0.05$; and ##, $P < 0.01$ *vs.* aldosterone-secreting adenomas.

Discussion

Based on the experimental evidence that ACTH receptor expression is down-regulated in adrenal malignancies (3, 4), the ACTH receptor gene is viewed as a hypothetical tumor suppressor gene. The recently identified seladin-1/DHCR24 gene has been found to be positively regulated by ACTH (5). Sarkar *et al.* (5) described seladin-1/DHCR24 overexpression in cortisol-secreting adenomas compared with the atrophic adjacent gland. However, in that study, a quantitative PCR technique was not used, and no other adrenocortical tumors were studied.

In our study, for the first time, we measured the levels of seladin-1/DHCR24 mRNA in different nodular lesions of the adrenal cortex by quantitative real-time RT-PCR. Our results indicate that the levels of expression do not significantly differ among the normal adrenal cortex and both cortisol- and aldosterone-secreting adenomas. Conversely, markedly reduced seladin-1/DHCR24 mRNA levels were detected in adrenocortical carcinomas, and the lowest levels were found in advanced disease (stage III–IV). The reduced levels of seladin-1/DHCR24 transcript in adrenal malignancies were confirmed by Western blot analysis of seladin-1/DHCR24 protein. Whether this finding, as well as previously reported observations regarding the ACTH receptor (3, 6), has relevance in the pathogenesis of adrenal cancer cannot be stated at present. It has been demonstrated previously that seladin-1/DHCR24 expression is modulated via the cAMP-dependent pathway, and *in vivo* experiments using animal models indicated that ACTH effectively stimulates seladin-1/DHCR24 production (5). We now demonstrated for the first time that ACTH increases seladin-1/DHCR24 expression in cultured cells from human adrenal cortex. The ACTH-mediated modulation of seladin-1/DHCR24 expression appeared to be supported by the finding that, in aldosterone-secreting adenomas in which the amount of expression of the ACTH receptor was significantly higher than in cortisol-secreting adenomas, carcinomas, and normal adrenal cortex, according to a previous report (15), the mean levels of seladin-1/DHCR24 protein appeared significantly higher, too. Therefore, reduced seladin-1/DHCR24 expression in adrenal cancer might be the result of concomitant reduced ACTH receptor expression, leading to impaired cAMP-dependent signaling. Accordingly, we have previously described loss or reduced levels of expression of the cAMP-dependent transcription factors cAMP response element binding protein and inducible cAMP early repressor in adrenal and thyroid

malignancies (4, 16). Altogether, these observations suggest that alterations of the ACTH-cAMP driven machinery may be regarded as markers of loss of cell differentiation. With regard to this point, it is worth mentioning that seladin-1/DHCR24 gene is involved in the synthesis of cholesterol, the precursor of steroid hormones, actively produced by the adrenal cortex (10). On the other hand, seladin-1/DHCR24 has been proven to have antiapoptotic properties and to inhibit caspase 3 activity in neuronal cells (9). Furthermore, seladin-1/DHCR24 expression has been inversely correlated with apoptotic death in the adrenal cortex as well (5). Reduced expression of a protein, such as seladin-1/DHCR24, which inhibits the activity of the mediator of apoptosis caspase 3, in cancer might be surprising. However, increased proliferation rate in tumoral cells may lead to increased apoptotic death as a consequence. Interestingly, increased caspase 3 activity has been demonstrated in breast cancer compared with both benign lesions and normal breast tissues (17–19). Nevertheless, both mitotic and apoptotic indexes appeared elevated in breast cancer, and the mitotic/apoptotic index was definitively increased (20, 21). Interestingly, although there are no reports on caspase activity in adrenal neoplasms, there is evidence that both the proliferation (MIB-1 index) and the apoptotic rate (bcl-2 and p53 expression, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling analysis) may be altered in carcinomas of the adrenal cortex (22–25). In our study, we found a significantly higher MIB-1 index in adrenocortical carcinomas (8.63%, mean value of Ki67-positive nuclei) than in adenomas (1.35% and 1.15% in cortisol- and aldosterone-secreting adenomas, respectively), in agreement with the previously mentioned reports, in which a cutoff of 5% well discriminated benign from malignant tumors. With regard to bcl-2, no benign or malignant tumor showed positivity, according to a cutoff of 10% of immunoreactive cells, as observed in other studies (22, 25). Parameters related to cell proliferation and apoptosis have been suggested as markers of malignancy together with other markers, such as the adrenal 4 binding protein (26), c-Myc (27), IGF-II (28), and telomerase activity (29). However, none of these markers has been shown to be able to predict malignancy in the single tumor with accuracy. In view of our results, we suggest seladin-1/DHCR24 as an additional potential marker of malignancy in the adrenal cortex, especially in cases where histopathology results are inconclusive. An index related to apoptosis, such as seladin-1/DHCR24, might potentially have additional implications. In fact, apoptosis-inducing drugs have been shown to be effective in inhibiting adrenal cancer cell proliferation *in vitro* (30–32). It is worth mentioning that apoptotic rate appears to be a good marker for predicting a positive response to chemotherapy, as observed in breast cancer cells, in which enforced activation of caspase 3 markedly improved the effect of drug treatment (33, 34). In the clinical setting, several chemotherapeutic agents (*i.e.* etoposide, doxorubicin, and cisplatin) have been used in advanced adrenal cancer disease, with some positive results in a subset of patients (35, 36). However, the efficacy of chemotherapy in the treatment of adrenal cancer remains largely unsatisfactory. Because the selection of the patients to be treated with cytotoxic agents might be critical for the success of the treatment, the caspase 3 inhibitor seladin-1/DHCR24 might be a new potentially useful tool for predicting response or resistance to chemotherapeutic agents.

In summary, in the present study, we have shown for the first time that the expression of the newly discovered antiapoptotic factor seladin-1/DHCR24 is reduced in adrenocortical carcinoma. Because of the possible prognostic and therapeutic implications of this finding, further investigation on the role of seladin-1/DHCR24 in regulating cell behavior and survival should be undertaken by assessing, for instance, the biological effects of enforced or silenced gene expression in *in vitro* systems.

Acknowledgments

Received June 23, 2003. Accepted December 8, 2003.

Address all correspondence and requests for reprints to: Alessandro Peri, M.D., Ph.D., Endocrine Unit, Department of Clinical Physiopathology, University of Florence, Viale Pieraccini, 6, 50139 Florence, Italy. E-mail: a.peri@dfc.unifi.it.

This work was partially supported by a grant from Associazione Italiana per la Ricerca sul Cancro.

References

1. **Simpson ER, Waterman MR** 1988 Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu Rev Physiol 50:427–440
2. **Koch CA, Pacak K, Chrousos GP** 2002 The molecular pathogenesis of hereditary and sporadic adrenocortical and adrenomedullary tumors. J Clin Endocrinol Metab 87:5367–5384
3. **Reincke M, Mora P, Beuschlein F, Arlt W, Chrousos GP, Allolio B** 1997 Deletion of the adrenocorticotropin receptor gene in human adrenocortical tumors: implications for tumorigenesis. J Clin Endocrinol Metab 82:3054–3058
4. **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, Mannelli M** 2001 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
5. **Sarkar D, Imai T, Kambe F, Shibata A, Ohmori S, Siddiq A, Hayasaka S, Funahashi H, Seo H** 2001 The human homolog of Diminuto/Dwarf1 gene (hDiminuto): a novel ACTH-responsive gene overexpressed in benign cortisol-producing adrenocortical adenomas. J Clin Endocrinol Metab 86:5130–5137
6. **Klahre U, Noguchi T, Fujioka S, Takatsuto S, Yokota T, Nomura T, Yoshida S, Chua NH** 1998 The Arabidopsis DIMINUTO/DWARF1 gene encodes a protein involved in steroid synthesis. Plant Cell 10:1677–1690
7. **McMorris TC** 1997 Recent developments in the field of plant steroid hormones. Lipids 32:1303–1308
8. **Bishop GJ, Nomura T, Yokota T, Harrison K, Noguchi T, Fujioka S, Takatsuto S, Jones JD, Kamiya Y** 1999 The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. Proc Natl Acad Sci USA 96:1761–1766
9. **Greeve I, Hermans-Borgmeyer I, Brellinger C, Kasper D, Gomez-Isla T, Behl C, Levkau B, Nitsch RM** 2001 The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. J Neurosci 20:7345–7352
10. **Waterham HR, Koster J, Romeijn GJ, Hennekam RC, Vreken P, Andersson HC, FitzPatrick DR, Kelley RI, Wanders RJ** 2001 Mutations in the 3β-hydroxysterol Δ24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am J Hum Genet 69:685–694
11. **Munari-Silem Y, Lebrethon MC, Morand I, Rousset B, Saez JM** 1995 Gap junction-mediated cell-to-cell communication in bovine and human adrenal cells. A process whereby cells increase their responsiveness to physiological corticotropin concentrations. J Clin Invest 95:1429–1439
12. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
13. **Bustin SA** 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39
14. **Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin S, Orlando C** 2002 Quantitative real-time reverse transcription polymerase chain reaction normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 309:293–300
15. **Arnaldi G, Mancini V, Costantini C, Giovanetti, M, Petrelli M, Masini A,**

Bertagna X, Mantero F 1998 ACTH receptor mRNA in human adrenocortical tumors: overexpression in aldosteronomas. Endocr Res 24:845–849
16. **Luciani P, Buci L, Conforti B, Tonacchera M, Elisei R, Vivaldi A, Cioppi F, Biliotti G, Manca G, Vitti P, Serio M, Peri A** 2003 Expression of cAMP response element-binding protein and sodium iodide symporter in benign non-functioning and malignant thyroid tumors. Eur J Endocrinol 148:579–586
17. **Vakkala M, Pääkö P, Soini Y** 1999 Expression of caspases 3, 6, and 8 is increased in parallel with apoptosis and histological aggressiveness of the breast lesion. Br J Cancer 81:592–599
18. **Wong SCC, Chan JKC, Lee KC, Hsiao WLW** 2001 Differential expression of p16/p21/p27 and cyclin D1/D3, and their relationships to cell proliferation, apoptosis, and tumor progression in invasive ductal carcinoma of the breast. J Pathol 194:35–42
19. **O'Donovan N, Crown J, Stunnel H, Hill ADK, McDermot E, O'Higgins N, Duffy MJ** 2003 Caspase 3 in breast cancer. Clin Cancer Res 9:738–742
20. **Mommers ECM, van Diest PJ, Leonhart AM, Meijer CJLM, Maak JPA** 1999 Balance of cell proliferation and apoptosis in breast carcinogenesis. Breast Cancer Res Treat 58:163–169
21. **Zhao H, Morimoto T, Sasa M, Tanaka T, Izumi K** 2002 Immunohistochemical expression of uPA, PAI-1, cathepsin D and apoptotic cells in ductal carcinoma *in situ* of the breast. Breast Cancer 9:118–126
22. **McNicol AM, Struthers AL, Nolan CE, Hermans J, Haak HR** 1997 Proliferation in adrenocortical tumors: correlation with clinical outcome and p53 status. Endocr Pathol 8:29–36
23. **Wachenfeld C, Beuschlein F, Zwermann O, Mora P, Fassnacht M, Allolio B, Reincke M** 2001 Discerning malignancy in adrenocortical tumors: are molecular markers useful? Eur J Endocrinol 145:335–341
24. **Bernini GP, Moretti A, Viacava P, Bonadio AG, Iacconi P, Miccoli P, Salvetti A** 2002 Apoptosis control and proliferation marker in human normal and neoplastic adrenocortical tissues. Br J Cancer 86:1561–1565
25. **Stojadinovic A, Brennan MF, Hoos A, Omeroglu A, Leung D, Dudas ME, Nissan A, Cordon-Cardo C, Ghossein R** 2003 Adrenocortical adenoma and carcinoma: histopathological and molecular comparative analysis. Mod Pathol 16:742–751
26. **Sasano H, Shizawa S, Suzuki T, Takayama K, Fukaya T, Morohashi K, Nagura H** 1995 Transcription factor adrenal 4 binding protein as a marker of adrenocortical malignancy. Hum Pathol 26:1154–1156
27. **Suzuki T, Sasano H, Nisikawa T, Rhame J, Wilkinson DS, Nagura H** 1992 Discerning malignancy in human adrenocortical neoplasms: utility of DNA flow cytometry and immunohistochemistry. Mod Pathol 5:224–231
28. **Gicquel C, Le Bouc Y** 1997 Molecular markers for malignancy in adrenocortical tumors. Horm Res 47:269–272
29. **Mannelli M, Gelmini S, Arnaldi G, Becherini L, Bemporad D, Crescioli C, Pazzagli M, Mantero F, Serio M, Orlando C** 2000 Telomerase activity is significantly enhanced in malignant adrenocortical tumors in comparison to benign adrenocortical adenomas. J Clin Endocrinol Metab 8:468–470
30. **Fallo F, Pilon C, Barzon L, Pistorello M, Pagotto U, Altavilla G, Boscaro M, Sonino N** 1996 Effects of taxol on the human NCI-H295 adrenocortical carcinoma cell line. Endocr Res 22:709–715
31. **Fallo F, Pilon C, Barzon L, Pistorello M, Pagotto U, Altavilla G, Boscaro M, Sonino N** 1998 Paclitaxel is an effective antiproliferative agent on the human NCI-H295 adrenocortical carcinoma cell line. Chemotherapy 44:129–134
32. **Villa R, Orlandi L, Berruti A, Dogliotti L, Zaffaroni N** 1999 Modulation of cytotoxic drug activity by mitotane and lonidamine in human adrenocortical carcinoma cells. Int J Oncol 14:133–138
33. **Blanc C, Deveraux QL, Krajewski S, Janicke RU, Porter AG, Reed JC, Jaggi R, Marti A** 2000 Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res 60:4386–4390
34. **Yang X-H, Sladek TL, Liu X, Butler BR, Froelich CJ, Thor AD** 2001 Reconstitution of caspase 3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. Cancer Res 61:348–354
35. **Berruti A, Terzolo M, Pia A, Angeli A, Dogliotti L** 1998 Mitotane associated with etoposide, doxorubicin, and cisplatin in the treatment of advanced adrenocortical carcinoma. Italian Group for the Study of Adrenal Cancer. Cancer 83:194–200
36. **Ahlman H, Khorram-Manesh A, Jansson S, Wangberg B, Nilsson O, Jacobsson CE, Lindstedt** 2001 Cytotoxic treatment of adrenocortical carcinoma. World J Surg 25:927–933

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.