EXPERIMENTAL STUDY

Analysis by cDNA microarrays of gene expression patterns of human adrenocortical tumors

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Abstract

Objectives: Adrenocortical carcinoma (ACC) is a rare malignant neoplasm with extremely poor prognosis. The molecular mechanisms of adrenocortical tumorigenesis are still not well understood. The comparative analysis by cDNA microarrays of gene-expression patterns of benign and malignant adrenocortical tumors allows us to identify new tumor-suppressor genes and proto-oncogenes underlying adrenocortical tumorigenesis.

Design and methods: Total RNA from fresh-frozen tissue of 10 ACC and 10 benign adrenocortical adenomas was isolated after histologic confirmation of neoplastic cellularity of at least 85%. The reference consisted of pooled RNA of 10 normal adrenal cortex samples. Amplified RNA of tumor and reference was used to synthesize Cy3- and Cy5-fluorescently labeled cDNA in a flip-color technique. D-chips containing 11 540 DNA spots were hybridized and scanned and the images were analyzed by ImaGene 3.0 software.

Results: The comparative analysis of gene expression revealed many genes with more than fourfold expression difference between ACC and normal tissue (42 genes), cortical adenoma and normal tissue (11 genes), and ACC and cortical adenoma (21 genes) respectively. As confirmed by real-time PCR, the IGF2 gene was significantly upregulated in ACCs versus cortical adenomas and normal cortical tissue. Genes that were downregulated in adrenocortical tumors included chromogranin B and early growth response factor 1.

Conclusions: Comprehensive expression profiling of adrenocortical tumors by the cDNA microarray technique is a very powerful tool to elucidate the molecular steps associated with the tumorigenesis of these ill-defined neoplasms. To evaluate the role of identified genes, further detailed analyses, including correlation with clinical data, are required.

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Introduction

Adrenal masses are a common disorder, affecting 3–7% of the population. Most turn out to be benign adrenocortical adenomas, which may be functional or nonfunctional. Much more rarely, these masses represent primary adrenal carcinoma (1). Adrenocortical carcinoma (ACC) is a highly malignant tumor with an incidence of ~1 per 1.7 million inhabitants per year in the West. Although ACC is rare, its highly aggressive behavior and 5-year survival rate of only 10–20% urgently require the decoding of its molecular basis to develop new strategies for diagnosis and treatment (2–6). The genetic background of adrenocortical tumorigenesis is poorly characterized. In other endocrine tissues, such as thyroid, there is conclusive evidence that hyperplasia and adenomas can precede

cancer. In the adrenal, there are clinical cases of either hyperplasia or adenoma associated with later development of cancer. However, only a few studies have attempted to characterize this process on a molecular basis (7). Although it is unclear whether there is an adenoma-carcinoma sequence, common patterns seen in adenomas and carcinomas, and the accumulation of chromosomal imbalances with tumor progression support the existence of an adenoma-carcinoma sequence (8). X-chromosome inactivation analysis has shown that ACCs are of monoclonal origin, whereas benign adenomas may be monoclonal or polyclonal (9–11). The evidence gathered so far shows that the transition from adrenal adenoma to carcinoma involves a monoclonal proliferation of cells that, among other yet to be characterized defects, have undergone chromosomal duplication at the

11p15.5 locus, leading to overexpression of the insulin-like growth factor (IGF)2 gene and abrogation of expression of the CDKN1C and H19 genes (12, 13). TP53 has been shown to be involved in progression to carcinoma in a subset of patients, and it has been suggested that the frequency of adrenocorticotrophic hormone (ACTH) receptor deletion might also be involved (1). Other key oncogenes and tumor suppressor genes remain to be identified. However, a recent study has reported that chromosomal loci 1p, 2p16, 11q13 and 17p may harbor potential tumor-suppressor genes, and chromosomes 4, 5 and 12 potential oncogenes associated with adrenal tumorigenesis (1). Therefore, detailed analysis of the genes involved is highly desirable. The development of the cDNA microarray technique offers the opportunity to analyze a large number of genes, and allows comparative analysis of gene-expression profiling in benign and malignant adrenocortical tumors and the identification of tumor-suppressor genes and proto-oncogenes associated with the initiation and progression of adrenocortical tumors. Findings from these analyses might clarify adrenocortical tumorigenesis and lead to the establishment of new diagnostic and prognostic markers as well as the characterization of novel strategies for treatment. Therefore, we have performed a comprehensive and representative analysis of neoplastic and nonneoplastic adrenocortical tissue samples. We were particularly interested in evaluating the differential profile of 11 500 genes with established importance for development and progression of malignant diseases (1), to assess whether combinations of genes can predict malignant tumors (2) and to validate the results by real time RT-PCR of selected candidate genes (3).

Materials and methods

Normal and tumor samples

The adrenocortical tissues analyzed in this study were obtained from the collection of fresh frozen adrenal tissue of the Department of Surgery, Philipps-University Marburg, Germany, collected between 1996 and 2003. For the purpose of this study, 10 ACCs and 10 adrenocortical adenomas (four from Conn's syndrome, four from Cushing's disease and two nonfunctional adenomas), as well as 10 nonneoplastic adrenal cortical tissue samples, were evaluated. The classification was determined by both conventional histologic methods (14) and Weiss score (15) where adenomas met fewer than four and carcinomas at least four of the criteria. The ethics committee of the university approved this study, and all patients participating in the study consented to sampling. Frozen tumor samples were formalin-fixed and embedded in paraffin, and sections were evaluated by hematoxylin and eosin (HE) staining regarding diagnosis and neoplastic cellularity. Only tumor samples with neoplasticity of greater than 85% were included

in the analysis. Control samples consisted of 10 histologically confirmed normal adrenal cortices.

RNA isolation was performed as follows. Frozen control and tumor tissue samples were dissected by the pathologist (A M) and homogenized in the presence of TRIzol Reagent (Invitrogen, Karlsruhe, Germany) by the manufacturer's protocol. Total RNA was then further purified by digestion with DNase I and recovery of RNA with the RNeasy kit (Qiagen, Hilden, Germany) by the supplier's protocol. To determine the integrity of RNA, standard RT-PCR for the amplification of 17 α -hydroxylase and β -actin was performed on 25 ng RNA with Qiagen's OneStep RT-PCR kit by the manufacturer's protocol. The primer sequences were as follows:

- Cyp17_forward TCTCTTGCTGCTTACCCTAG;
Cyp17_reverse TCAAGGAGATGACATTGGTT
(GenBank accession no. NM_000102).
- β -actin_forward GATGATGATATCGCCGCGCTCG-TCGTC; β -actin_reverse GTGCCTCAGGCAGCG-GAACCGCTCA (GenBank accession no. M10277).

The reaction mixture was incubated at 50 °C for 30 min and 95 °C for 15 min, followed by 30 cycles of standard PCR (1-min denaturation at 95 °C, 1-min annealing at 58 °C and 1-min extension at 72 °C). PCR products were visualized by ethidium bromide staining of PAGE. Only samples showing expression of Cyp17 as well as β -actin were included in analysis. RNA (2 μ g) from 10 ACC and 10 adenomas (eight functional and two non-functional adenomas) was amplified with the MessageAmp aRNA Kit (Ambion, Huntingdon, UK). For the reference, the 10 control samples were amplified and then pooled. Amino allyl-cDNA was synthesized with 2 μ g aRNA and then labeled and purified with the CyScribe Post-Labeling Kit (Amersham Biosciences, Freiburg, Germany). Samples were fluorescently labeled with Cy3 and Cy5 by the flip-color technique.

For gene-expression profiling, the reference and tumor samples were mixed, denatured and then hybridized to microarrays for 16 h at 56 °C and washed at a stringency of 0.1% SSC and 0.1% SDS. The microarray contains 11 540 DNA spots; detailed protocols and data description of the chip are available from the website: www.imt.uni-marburg.de. Each experiment was performed as a sandwich hybridization with two arrays. Spot intensities were extracted from a scanned image with Image 3.0 Software (BioDiscovery, Los Angeles, CA, USA). For each spot, median signal and background intensities for both channels were obtained. To account for spot differences, the background-corrected ratios of the two channels were calculated and log2-transformed. To balance the fluorescence intensities for the two dyes as well as to allow comparison of expression levels across experiments, the raw data were standardized. We used a spatial and intensity-dependent standardization (like Yang *et al.* (16)) to correct for inherent bias on

each chip (the lowest scatter-plot). As each gene was measured twice in the sandwich hybridization, mean log-ratios M were calculated from replicates. If gene replicates differed more than the maximum of threefold and 75% of the calculated average log-ratio, or the background intensity was higher than signal intensity, the spot was excluded on that array. Differentially expressed genes were selected by a fold-change difference of at least 2 and an absolute value of the t -statistic of 1.96. Prior to the cluster analysis, the expression profile of each gene was centered by subtracting the mean observed value. Average linkage hierarchic clustering was then performed for genes as well as for chips with the Euclidean distance metric as implemented in the program Genesis (17).

The microarray results were validated for three candidate genes known to be expressed in adrenocortical tissue, including chromogranin B (CgB), early growth response gene 1 (Egr-1), and IGF2 by real-time RT-PCR analysis. For validation, 10 μ g total RNA were reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and an oligo dT15 primer, according to the manufacturer's instructions and previously published methods (18). Real-time PCR with the LightCycler System (Roche, Mannheim, Germany) was performed in a reaction mixture of 20 μ l using the QuantiTect SYBR Green PCR Kit according to the manufacturer (Qiagen). Primers designed for analysis were as follows:

- Egr1 (GenBank accession no. NM001964) forward CGAGCAGCCCTACGAGCACCTGAC and reverse TGCGCAGCTCAGGGGTGGGCTCTG.
- IGF2 (GenBank accession no. BC000531) forward CCGTGCTTCCGGACAACT and reverse GGACTGCTTCCAGGTGTCATATT.
- CgB (GenBank accession no. AL035461) forward TGCCAGTGGATAACAGGAAC and reverse TCTTCAGGACTTGGCGGCA.
- GAPDH forward CGTCTTACCACCATGGAGA and reverse CGGCCATCACGCCACAGTTT.

The cycle threshold values for each gene were analyzed relative to those for GAPDH.

Results

For this study, 10 adrenocortical cancers, as established by histology, and 10 adenomas, of which eight were functional, including four aldosterone-producing adenomas, four cortisol-producing adenomas, and two nonfunctional adenomas (incidentalomas), were selected from the tissue bank of the Department of Surgery, Philipps-University Marburg. The clinicopathologic data of the adrenocortical tumors analyzed are summarized in Table 1. Normal adrenal cortex, adrenocortical adenoma and ACC were analyzed histologically

Table 1 Pathologic and clinical aspects of adrenocortical cases used for profiling studies.

Designation	Tissue type	Age	Gender	Stage*	Syndrome	Metastatic sites**	Follow-up
A1	ACC	50	F	II	None	None	DOD
A2	ACC	46	F	I	None	None	NED
A3	ACC	59	F	III	None	None	NED
A4	ACC	43	F	IV	Cushing	Lung	DOD
A5	ACC	15	M	II	Cushing	None	DOD
A6	ACC	74	F	III	None	None	DURC
A7	ACC	52	M	III	None	None	DOD
A8	ACC	51	F	III	Cushing, AGS	None	DOD
A9	ACC	37	F	II	Conn	None	NED
A10	ACC	37	F	III	Cushing	None	AWD
A11	ACC	84	F	II	None	None	NED
A12	ACC	43	M	II	None	None	DOD
A15	ACC	17	F	III	Cushing, AGS	None	NED
T1	Adenoma	46	M	N.A.	Conn		NED
T2	Adenoma	45	F	N.A.	Conn		NED
T3	Adenoma	47	F	N.A.	Conn		NED
T4	Adenoma	54	M	N.A.	Conn		NED
T5	Adenoma	65	M	N.A.	Cush		NED
T6	Adenoma	27	F	N.A.	Cush		NED
T7	Adenoma	47	F	N.A.	Cush		NED
T8	Adenoma	45	F	N.A.	Cush		NED
T9	Adenoma	65	F	N.A.	Inc		NED
T10	Adenoma	52	F	N.A.	Inc		NED
T11	Adenoma	64	M	N.A.	Inc		NED
T12	Adenoma	73	F	N.A.	Inc		NED

*Tumors graded according to UICC.

**At time of operation.

Cush: Cushing's disease; Inc: Incidentaloma; N.A.: not applicable; Conn: Conn's syndrome; AGS: adrenogenital syndrome; DOD: death of disease; NED: no evidence of disease; AWD: alive with disease; DURC: death of unrelated cause.

to confirm tissue origin, lack of necrosis and, for tumors, neoplasticity of greater than 85%, before proceeding with nucleic acid purification. The normal tissue displayed regularly shaped nuclei and fat content. Fig. 1 shows representative examples of HE staining of tissue samples used for the RNA purification. The adrenal origin of the samples was additionally ensured by the RNA expression of 17 α -hydroxylase (Cyp17), in addition to β -actin, by RT-PCR. All samples chosen for further analysis were positive for both Cyp17 and

Figure 1 HE staining of normal adrenal cortex (A), adenoma (B) and adrenocortical carcinoma (C). $\times 100$.

β -actin (data not shown). Microarray analysis demonstrated that ACCs were more dissimilar to normal adrenal than adenomas. The adenomas were more closely related to each other and to normal adrenal; this is not an entirely unexpected result given the histologic similarity of the tissues. In particular, 40 differentially expressed genes were found in adenomas in comparison to normal adrenal (Table 2), and 144 differentially expressed genes were detected in ACCs (Table 3). As shown in Fig. 2 and Table 4, more than 60 genes were found with at least a threefold change in mRNA levels. Both up- and downregulated genes were identified (Table 4). These genes, with at least threefold differences in mRNA expression, were then subjected to cluster analysis. Transcriptional profiles, which distinguish between benign and malignant adrenocortical tumors, identified several differentially expressed transcripts as demonstrated by cluster analysis (Fig. 2). The sample dendrogram revealed the similarities among the adenomas (T) and the carcinomas (A) respectively, and clearly distinguished the two groups. Two major clusters of genes were differentially expressed in the carcinomas compared with the adenomas: genes that were expressed at a higher level in the carcinomas and those that were expressed at a lower level in the carcinomas. The former include the gene for IGF-2 and potential oncogenes. The latter represent potential tumor-suppressor genes.

Some of the differentially expressed genes were chosen for further analysis to ensure the validity of the microarray results. As expected from the literature (1, 12, 19), the IGF-2 gene expression was 3–7-fold greater in ACCs than in adenomas. Real-time RT-PCR confirmed this result where the cycle threshold crossing point (CT) for the ACC samples was 3–5 values less, representing a maximal increase in expression of 8–32-fold in comparison to the control or adenoma samples (Fig. 3).

As one of our main interests is the identification of potential tumor-suppressor genes whose investigation may contribute to the understanding of the pathomechanism of ACC, we decided to concentrate in our initial analysis on clearly downregulated genes. The tissue-specifically expressed gene, chromogranin B (CgB), was found to be downregulated in both adenomas (28-fold) (Table 2) and ACCs (13-fold) (Table 3). The loss of a tissue-specifically expressed gene could reflect dedifferentiation of tumor tissue. This decrease in expression was confirmed by real-time RT-PCR, which demonstrated an increase in the CT value from 4 to 5, representing at most a 16–32-fold decrease in expression, for the adenomas analyzed, and from 3 to 4, or at most a 8–16-fold decrease for the ACCs.

The Egr-1 gene was downregulated in ACC in comparison to normal adrenal by eightfold (Fig. 3). This finding was also confirmed by real-time RT-PCR, where the CT values for the ACCs were increased by

Table 2 List of genes found to be differentially regulated in adenomas relative to normal adrenal cortical tissue in microarray analysis.

Gene name	Accession no.	Fold regulation	Up/down
Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2	H08850	4.42	up
Chemokine (C-C motif) ligand 2	AA425102	3.94	up
Activity-regulated cytoskeleton-associated protein	H86117	3.84	up
Hypothetical protein FLJ10052	R54822	3.76	up
Purkinje cell protein 4	AA452826	3.36	up
Mucolipin 3	AA171718	3.29	up
Neuronal pentraxin II	AA683041	2.40	up
Cytoplasmic FMR1 interacting protein 2	H12044	2.38	up
B-cell CLL/lymphoma 11B (zinc finger protein)	H09748	2.27	up
Chromogranin B (secretogranin 1)	W37769	28.46	down
Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	AA457042	12.08	down
EST	H11453	9.86	down
yz80b09.s1 Soares_multiple_sclerosis_2NbHMSP <i>Homo sapiens</i> cDNA clone IMAGE:289337 3'	N92646	7.60	down
Chromosome 1 open reading frame 29	AA410188	7.54	down
Insulin-like growth factor binding protein 6	AA479428	7.35	down
Brain expressed, X-linked 1	W60582	4.70	down
Contactin 1	H19315	4.46	down
Dickkopf homolog 3 (<i>Xenopus laevis</i>)	AA425947	4.33	down
Sjogren syndrome antigen B (autoantigen La)	H29485	4.08	down
Chemokine (C-C motif) ligand 2	AA938563	3.99	down
Cathepsin H	AA487231	3.93	down
Sodium channel, voltage-gated, type III, beta	R53930	3.82	down
Insulin-like growth factor binding protein 6	AA478724	3.71	down
Heat shock 105 kDa/110 kDa protein 1	AA485151	3.71	down
<i>Homo sapiens</i> cDNA FLJ38885 fis, clone MESAN2017417	T89094	3.63	down
Sodium channel, voltage-gated, type III, beta	AA134824	3.55	down
Solute carrier family 40 (iron-regulated transporter), member 1	T52564 T57235	3.43	down
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	AA670438	3.35	down
KIAA1576 protein	AA609348	3.34	down
Protein tyrosine phosphatase, receptor type, N polypeptide 2	AA464590	3.30	down
Sal-like 2 (<i>Drosophila</i>)	H23254	3.29	down
Connective tissue growth factor	AA598794	3.16	down
Complexin 2	H09966	3.10	down
Integral membrane protein 2C	AA034213	3.08	down
Hypothetical protein FLJ39155	R08141	3.07	down
Calmodulin 1 (phosphorylase kinase, delta)	R76554 R76277	3.02	down
<i>Homo sapiens</i> cDNA FLJ39226 fis, clone OCBBF2007232.	H09078	2.99	down
Aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	R93124	2.95	down
gr04f12.s1 soares fetal liver spleen 1NFLS <i>Homo sapiens</i> cDNA clone IMAGE: 204335	H59916	2.92	down
Nucleolar protein 4	AA430033	2.87	down

4, representing a maximal decrease in expression of up to 16-fold. Thus, for each of these candidate genes, the differential gene expression was confirmed by real-time RT-PCR, and the findings were comparable to those of the microarray analysis (Fig. 3).

Discussion

DNA microarray technology allows comprehensive examination of the transcriptional profile of tumors. It is rapidly being applied to various problems in pathology and oncology, such as tumor classification, and it is a useful gene discovery tool to complement other similar technologies. In this study, we used DNA microarrays

to generate transcriptional profiles of benign and malignant adrenocortical tumors with various hormonal secretion profiles (Table 1). We demonstrated for the first time that these profiles can distinguish normal and benign tissues from malignant tumors and identify differentially expressed genes that may help to explain the pathogenesis of the disease and have diagnostic and therapeutic implications. Surprisingly, only 11 genes were found to be differentially expressed more than fourfold in comparing adenomas to normal tissue (Table 2). A comparison of the ACCs to normal tissue resulted in a list of 42 genes that were differentially expressed more than fourfold (Table 3), and the comparison ACCs to adenomas resulted in 21 genes that were more than fourfold differentially expressed.

Table 3 List of genes found to be differentially regulated in ACC relative to normal adrenal cortical tissue in the microarray analysis.

Gene name	Accession no.	Fold regulation	Up/down
Dopachrome tautomerase (dopachrome delta-isomerase. tyrosine-related protein 2)	AA478553	6.14	up
Human neuropeptide Y receptor Y1 (NPYY1)	R43817	6.11	up
Sodium channel, voltage-gated, type III, beta	R53930	5.78	up
Fibronectin 1	R62612	4.43	up
Chemokine (C-C motif) ligand 2	AA938563	4.30	up
Sjogren syndrome antigen B (autoantigen La)	H29485	4.29	up
Fibronectin 1	R62612	4.28	up
Insulin-like growth factor 2 (somatomedin A)	N54596	4.16	up
GA binding protein transcription factor. alpha subunit 60 kDa	H96241	4.12	up
Hypothetical protein MGC5306	H47257	4.07	up
EST	N59772	3.93	up
Hydroxy-delta-5-steroid dehydrogenase. 3 betaand steroid delta-isomerase 1	R68803	3.84	up
Dickkopf homolog 3 (<i>Xenopus laevis</i>)	AA425947	3.77	up
Aldehyde dehydrogenase 1 family. member A3	AA455235	3.69	up
Clone IMAGE:120162 mRNA sequence	T95274	3.67	up
CDC28 protein kinase regulatory subunit 2.	AA397813	3.51	up
Chemokine (C-C motif) ligand 2	AA425102	3.27	up
EST	H16389	3.24	up
Insulin-like growth factor binding protein 3	AA598601	3.22	up
Immediate early response 3	AA457705	3.21	up
Dentatorubral-pallidolysian atrophy (atrophin-1)	H08643	3.20	up
Ectodermal-neural cortex (with BTB-like domain)	H72122	3.18	up
Clone DKFZp434E235	H22563	3.18	up
yq51h12.s1 Soares fetal liver spleen 1NFLS cDNA clone IMAGE:199367 3'..	R95691	3.11	up
Dopa decarboxylase (aromatic L-amino acid decarboxylase)	AA702640	3.11	up
Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	AA282134	3.03	up
Fibulin 2	AA452840	3.02	up
c-src tyrosine kinase	AA079775	2.98	up
Flotillin 1	AA488175	2.92	up
Fibroblast growth factor 14	AA400047	2.89	up
RWD domain containing 1	AA487499	2.89	up
Hypothetical protein FLJ37078	R44292	2.84	up
Sodium channel. nonvoltage-gated 1 alpha	AA458982	2.84	up
Troponin I, skeletal, fast	AA181334	2.82	up
Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase	AA478066	2.73	up
Insulin-like growth factor 2 (somatomedin A)	N54596	2.63	up
Parathymosin	R11526	2.53	up
Cadherin 13. H-cadherin (heart)	R41787	2.50	up
Adrenergic. alpha-2A-. receptor	T48692	2.49	up
Glucocorticoid receptor DNA binding factor 1	N72276	2.42	up
CDC28 protein kinase regulatory subunit 2	AA010065	2.41	up
Signal recognition particle receptor, B subunit	R95693	2.37	up
Cytochrome c oxidase subunit VIa polypeptide 1	AA482243	2.35	up
Ubiquitin-like 1 (sentrin)	AA488626	2.33	up
Collagen, type V, alpha 1	R75635	2.30	up
Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2. Pfeiffer syndrome)	R54846	2.27	up
Splicing factor. arginine/serine-rich 2. interacting protein	R91171	2.25	up
EST	T97592	2.23	up
Lymphocyte-specific protein 1	T90632	2.18	up
Likely ortholog of rat vacuole membrane protein 1	AA485373	2.14	up
Dishevelled. dsh homolog 2 (<i>Drosophila</i>)	R38325	2.13	up
Kruppel-like zinc finger protein GLIS2	R43826	2.12	up
Dehydrogenase/reductase (SDR family) member 7	H87144	2.12	up
Insulin-like growth factor 2 (somatomedin A)	N74623	2.11	up
Hypothetical protein BC013949	W69741	2.11	up
Dipeptidylpeptidase 4 (CD26. adenosine deaminase complexing protein 2)	W70234	2.11	up
Nucleoredoxin	T64216	2.10	up
Zinc finger protein 161	AA232647	2.10	up
Hypothetical protein from clone 643	T53404	2.09	up
BTG family. member 3	N52496	2.07	up
Enoyl Coenzyme A hydratase domain containing 1	AA173573	2.06	up
Glyceraldehyde-3-phosphate dehydrogenase	h52950	2.03	up
EST	H48467	2.03	up
Prefoldin 5	AA446453	2.03	up

Table 3 Continued

Gene name	Accession no.	Fold regulation	Up/down
Integrin, alpha 7	T60926	2.02	up
Thymopoietin	T63980	2.02	up
Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	AA457042	13.60	down
Chromogranin B (secretogranin 1)	W37769	12.72	down
Actin binding LIM protein 1	AA406601	11.43	down
EST	H11453	9.23	down
Ferredoxin 1	AA187349	8.96	down
FBJ murine osteosarcoma viral oncogene homolog B	T61948	8.32	down
yz80b09.s1 Soares_multiple_sclerosis_2NbHMSP Homo sapiens cDNA clone IMAGE:289337 3'	N92646	8.17	down
Early growth response 1	AA486533	7.54	down
EST	N68993	7.54	down
Fatty acid binding protein 5 (psoriasis-associated)	T60075	7.49	down
Aldehyde dehydrogenase 1 family, member A1	AA664101	7.35	down
zb50h07.s1 Soares_fetal_lung_NbHL19W Homo sapiens cDNA clone IMAGE:307069 3'	N93686	7.08	down
Chromosome 1 open reading frame 29	AA410188	6.96	down
Contactin 1	H19315	6.79	down
Microsomal glutathione S-transferase 1	AA495936	6.58	down
Glucose phosphate isomerase	AA401111	6.54	down
Regucalcin (senescence marker protein-30)	H05140	6.28	down
Interferon-induced protein with tetratricopeptide repeats 1	AA489640	5.04	down
Chemokine (C-C motif) ligand 15	R96626	5.03	down
Solute carrier family 16 (monocarboxylic acid transporters) member 9	W16424	4.87	down
DKFZP586B1621 protein	H20543	4.85	down
High mobility group AT-hook 1	AI042404	4.80	down
Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1. (angioedema, hereditary)	AA481438	4.64	down
Mitogen-activated protein kinase kinase 5	AA150828	4.52	down
Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2	H08850	4.48	down
Vascular cell adhesion molecule 1	H07072	4.47	down
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	AA670438	4.31	down
v-jun sarcoma virus 17 oncogene homolog (avian)	W96155	4.30	down
Activity-regulated cytoskeleton-associated protein	H86117	4.26	down
Heat shock 105 kDa/110 kDa protein 1	AA485151	4.21	down
Glutathione S-transferase theta 1	H99813	4.21	down
Insulin-like growth factor binding protein 6	AA479428	4.08	down
Atp-binding cassette, sub-family B (MDR/TAP), member 1	AA459824	3.95	down
Insulin-like growth factor binding protein 6	AA478724	3.89	down
Zinc finger protein 145 (Kruppel-like, expressed in promyelocytic leukemia)	AA101632	3.88	down
Fibulin 1	AA134871	3.85	down
Chromosome 9 open reading frame 97	AA235388	3.84	down
KIAA1268 protein	T64956	3.82	down
Solute carrier family 26 (sulfate transporter), member 2	W15263	3.80	down
Major histocompatibility complex, class II, DP beta 1	AA486532	3.80	down
CD163 antigen	AA401693	3.79	down
Glutathione peroxidase 3 (plasma)	AA664180	3.79	down
MAX protein	T89496	3.76	down
Insulin-like growth factor 1 receptor	H13300	3.75	down
Arachidonate 5-lipoxygenase-activating protein	T49652	3.74	down
Interferon-induced transmembrane protein 1 (9-27)	AA058323	3.66	down
Chromosome 10 open reading frame 10	N55269	3.65	down
Scavenger receptor class B, member 1	AA443899	3.61	down
Connective tissue growth factor	AA598794	3.59	down
EST	T91100	3.57	down
Discoidin domain receptor family, member 2	AA243749	3.56	down
Homo sapiens cDNA FLJ40165 fis. clone TEST12015962.	H22854	3.50	down
Solute carrier family 40 (iron-regulated transporter), member 1	T52564 T57235	3.44	down
Hypothetical protein FLJ39155	R08141	3.41	down
Homo sapiens cDNA FLJ38885 fis. clone MESAN2017417.	T89094	3.40	down
Plasminogen-like	T67549	3.38	down
Periostin, osteoblast specific factor	AA598653	3.37	down
ATP-binding cassette, subfamily C (CFTR/MRP), member 3	AA429895	3.37	down
Chemokine-like factor superfamily 3	AA486561	3.36	down
ALEX3 protein	N54456	3.33	down

Table 3 Continued

Gene name	Accession no.	Fold regulation	Up/down
Interferon, alpha-inducible protein (clone IFI-6-16)	AA448478	3.29	down
Complement component 7	AA598478	3.27	down
KIAA1576 protein	AA609348	3.23	down
Sodium channel, voltage-gated, type III, beta	AA134824	3.11	down
Retinoic acid receptor responder (tazarotene induced) 2	AA482067	3.07	down
Complexin 2	H09966	3.05	down
Glutaredoxin (thioltransferase)	AA291163	3.03	down
ATP-binding cassette, subfamily B (MDR/TAP), member 1	AA135958	3.03	down
AE binding protein 1	AA490462	2.99	down
Hypothetical protein FLJ20637	AA487462	2.98	down
Fibronectin type III domain containing 5	N54901	2.92	down
Glutathione S-transferase A4	AA152347	2.91	down
G protein-coupled receptor 116	T63971	2.90	down
Calmodulin 1 (phosphorylase kinase, delta)	R76554 R76277	2.88	down
Major histocompatibility complex, class II; DR beta 5	AA485739	2.86	down
Scm-like with four mbt domains 1	AA400512	2.26	down
Notch homolog 3 (<i>Drosophila</i>)	T63511	2.22	down
Peroxisomal ion protease	T67138	2.04	down

The gene for chromogranin B (CgB) was found to be downregulated in both adenomas (28-fold) (Table 2) and ACCs (13-fold) (Table 3). This finding was confirmed by real-time PCR (Fig. 3). Chromogranins are representative proteins contained in endocrine cells of various organs, including some ductal cells of the breast. Homology between the BRCA1 protein (1214–1223) and the chromogranins has been detected, suggesting that chromogranin may play the role of a tumor suppressor, like BRCA1 (20). Our results suggest that loss of CgB may be an early event in adrenocortical tumorigenesis. In patients with lymph-node-negative primary invasive ductal breast carcinoma, CgB-negative tumors demonstrated a significantly poorer prognosis than in patients with CgB-positive tumors. In univariate analysis, a significantly increased risk of disease progression and death was present in patients with CgA-poor and CgB-poor tumors respectively (21). It was concluded that the CgB immunostaining pattern of the primary tumor can distinguish patients with increased risk of death in patients with sporadic medullary thyroid carcinoma. The problem of whether CgB represents a prognostic factor in ACCs requires a large-scale study.

Another interesting potential tumor suppressor is the Egr-1 gene. As validated by real-time PCR, it was downregulated in ACC in comparison to normal adrenal cortex tissue and adrenal adenoma by eightfold and threefold respectively (Fig. 3). Egr-1 is a transcription factor that has previously been suggested to be a master switch regulating inflammatory parameters (22). Egr-1 is also known as nerve growth factor induced-A (NGFI-A), Krox-24, ZIF268, ETR103 and TIS8. It is a phosphorylated zinc-finger-containing transcription factor often associated with cell growth stimulation (23). The gene for Egr-1 is located on the q31.1 'cytokine cluster' region of chromosome 5 in man, and is rapidly and transiently induced by a

large number of stressful stimuli as well as growth factors and cytokines. In fact, Egr-1 has been proposed as a master switch of gene expression underlying coordinated responses to various types of injury (23).

Apoptosis-regulating genes, such as TP53 and $\Delta Np73\alpha$, are known to increase the expression of Egr-1 (24, 25). Egr-1 has previously been implicated in the development and maintenance of prostate cancer (26, 27). Thus, further detailed, large-scale studies are warranted to clarify the role of Egr-1 in ACC.

One of the most interesting overexpressed genes is IGF2 on chromosome 11p15. Like other groups, we found a 3–7-fold overexpression of IGF2 in ACCs compared with adenomas and normal adrenal cortex (1, 13, 19). IGF-I and IGF-II are polypeptides involved in metabolism, growth and cell differentiation. They are synthesized in various tissues and have endocrine and auto/paracrine mechanisms of action depending on the tissue origin (2, 3). Both peptides are normally produced in adrenocortical cells (4, 19, 28). Through its action on steroidogenesis enzymes, IGF-I maintains the differentiated functions of the cell (19, 29). The precise role of IGF-II in mature adrenocortical gland is less clear. The IGF2 gene induction has previously been described as one of the most significant differences between ACCs and adrenal adenomas (1). Genetic alterations involving the 11p15 locus are very common in malignant tumors, but are found only in rare adrenal adenomas (13). The fact that we found this as well is further verification of our results. However, in adrenocortical tumors associated with Beckwith–Wiedemann syndrome, allelic losses at the 11p15 locus have been found in both adenomas and carcinomas as well as in familial carcinomas. Current data suggest that abnormalities in structure and/or expression of the IGF-II gene are a late event in the multistep tumorigenesis of sporadic adrenocortical neoplasms.

Figure 2 Hierarchic cluster analysis of differentially expressed genes in adrenal cortical adenomas and carcinomas. Both genes and individual tumors are clustered in this diagram. Green indicates downregulation of a gene in a given tumor; red indicates upregulation relative to the mean. Gray spots indicate missing data. Table 4 provides the fold regulation for each gene. The numbers at the top identify the individual tumors. Brackets above the tumors and on the right side of gene list indicate the clustering.

Several other genes were over- or underexpressed in ACCs compared with adrenocortical adenomas and normal adrenal cortex tissues, but we have not yet validated the expression pattern by real-time PCR. The presence of a gene on the list in Table 4 does not indicate that the gene product is either necessary or sufficient for causing ACC, but only that it is expressed as part of the complex pattern of gene expression that occurs during the initiation of the disease.

During the course of our studies, three other groups performed similar analyses of adrenocortical tissue (19, 30, 31). In the search for reliable markers for the

clinical management of adrenal tumors, de Fraipont *et al.* (31) designed an adrenal-specific microarray. They identified two clusters of genes, the IGF2 cluster and the steroidogenesis cluster, which, in combination, provide a good predictor of malignancy. As in our studies, they also confirmed the finding from the earlier analysis by Giordano *et al.* (19) demonstrating upregulation of IGF2 expression (30). Adrenal hyperplasia was analyzed in a similar fashion by Bourdeau *et al.* (30).

In summary, our findings indicate that microarray analysis can distinguish between ACC and adenomas by the differential expression of a set of the genes

Table 4 List of Genes found to be differentially regulated in ACC relative to adenomas in the microarray analysis^a.

Gene name	Accession no.	Fold regulation	Up/down
Cathepsin H	AA487231	3.81	up
Mucolipin 3	AA171718	4.34	up
Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	R54846	4.41	up
Aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	R93124	3.45	up
Fibronectin 1	R62612	3.73	up
Fibronectin 1	R62612	3.51	up
GABinding protein transcription factor, alpha subunit 60 kDa	H96241	3.12	up
Homo sapiens clone IMAGE:120162 mRNA sequence	T95274	3.24	up
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid deltaisomerase 1	R68803	5.67	down
yq51h12.s1 soares fetal liver spleen 1NFLS <i>Homo sapiens</i> cDNA clone	R95691	4.02	up
IMAGE:199367 3', mRNA sequence			
EST	T97592	3.26	up
Likely ortholog of rat vacuole membrane protein 1	AA485373	2.89	up
Periostin, osteoblast specific factor	AA598653	3.38	up
yg22a04.s1 Soares infant brain 1NIB <i>Homo sapiens</i> cDNA clone	R43910	3.09	up
IMAGE:32 962 3', mRNA sequence			
Cadherin 13; H cadherin (heart)	R41787	3.57	up
Insulin-like growth factor 2 (somatomedin A)	N54596	6.47	up
Ubiquitin-like 1 (sentrin)	AA488626	2.96	up
Insulin-like growth factor 2 (somatomedin A)	N54596	2.91	up
Hypothetical protein from clone 643	T53404	2.98	up
Parathymosin	R11526	2.89	up
Hypothetical protein MGC5306	H47257	2.93	down
Cytoplasmic FMR1 interacting protein 2	H12044	6.03	down
Purkinje cell protein 4	AA452826	3.10	down
Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	AA282134	3.08	down
zb50h07.s1 Soares_fetal_lung_NbHL19W <i>Homo sapiens</i> cDNA clone	N93686	9.65	down
IMAGE:307069 3', mRNA sequence			
Paralemmmin	H14208	3.55	down
Hyaluronoglucosaminidase 1	AA464196	3.41	down
ATP-binding cassette, sub-family B (MDR/TAP), member 1	AA459824	4.57	down
Putative membrane protein	T98298	3.22	down
Bone morphogenetic protein 1	R56774	3.18	down
Scavenger receptor class B, member 1	AA443899	6.23	down
Cytochrome p450, family 17, subfamily A, polypeptide 1	R16838	2.87	down
Chemokine (C-C motif) ligand 15	R96626	4.73	down
Retinoic acid receptor responder(tazarotene induced)2	AA482067	3.39	down
Glutathione S-transferase theta 1	H99813	2.95	down
FBJ murine osteosarcoma viral oncogene homolog B	T61948	6.82	down
Fatty acid binding protein 5 (psoriasis-associated)	T60075	3.38	down
Early growth response 1	AA486533	3.26	down
Immediate early response 3	AA457705	3.04	down
Arachidonate 5-lipoxygenase-activating protein	T49652	3.54	down
Chemokine-like factor super family 3	AA486561	3.19	down
Dopachrome tautomerase(dopachrome delta-isomerase, tyrosinerelated protein 2)	AA478553	2.88	down
Fibroblast growth factor 11	H15533	3.62	down
EST	N59772	6.40	down
High mobility group AT-hook 1	A1042404	3.73	down
Glutathione S-transferase A3	N30096	3.48	down
Likely ortholog of mouse cancer related gene- liver 2	H23137	3.13	down
Human neuropeptide Y receptor Y1 (NPYY1)	R43817	3.60	down
MAX protein	T89496	3.10	down
Glucose phosphate isomerase	AA401111	8.53	down
Microsomal glutathione S-transferase 1	AA495936	6.87	down
CD 163 antigen	AA401693	4.24	down
Transcribed sequence with moderate similarity to protein pir:A36563 (<i>H.sapiens</i>) A36563 mannose receptor precursor – human	H16389	3.28	down
Glutaredoxin (thioltransferase)	AA291163	3.09	down
Adlican	AA464691	3.50	down
Glutathione peroxidase 3 (plasma)	AA664180	3.08	down
Aldehyde dehydrogenase 1 family, member A3	AA455235	3.64	down
Fibulin 1	AA134871	2.88	down
Actin binding LIM protein 1	AA406601	5.91	down
Aldehyde dehydrogenase 1 family, member A1	AA664101	5.25	down

Table 4 Continued

Gene name	Accession no.	Fold regulation	Up/down
Glutathione S-transferase A4	AA152347	3.49	down
Hypothetical protein MGC35366	N59799	3.07	down
ATP-binding cassette, subfamily C (CFTR/MRP), member 3	AA429895	5.69	down
DKFZP586B1621 protein	H20543	3.14	down
Ferredoxin 1	AA187349	9.33	down
EST	T91100	2.94	down
Solute carrier family 26 (sulfate transporter), member 2	W15263	5.84	down
DKFZp586P1124	R41972	3.10	down
Delta-notch-like EGF repeat-containing transmembrane	H28681	2.95	down
Mitogen-activated protein kinase kinase kinase 5	AA150828	2.93	down
Regucalcin (senescence marker protein-30)	H05140	6.18	down
Homo sapiens cDNA FLJ40165fis, clone TEST12015962	H22854	3.87	down
Peroxisome oxidin 3	H19203	3.28	down
Interleukin 1 receptor, type 1	AA464526	2.84	down

^aListed in order of appearance in hierarchic cluster, Fig. 2.

Figure 3 Real-time RT-PCR. Shown is a comparison of the results of the microarray analysis and quantitative PCR experiments documenting the expression of the indicated genes. The plot shows the change in the level of expression found in the microarray analysis (□) and the change in the cycle-threshold value from control found in real-time RT-PCR (■) for each gene relative to GAPDH. ACC: adrenocortical carcinoma.

analyzed. Further analysis of the IGF2 gene validated the experimental design. Underexpression of the CgB gene was found in both types of neoplasms, and the Egr-1 gene was downregulated in the ACCs. Further detailed analyses are warranted to elucidate the role of these genes in adrenal tumorigenesis.

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References

- Sidhu S, Gicquel C, Bambach CP, Campbell P, Magarey C, Robinson BG & Delbridge LW. Clinical and molecular aspects of adrenocortical tumorigenesis. *Australian and New Zealand Journal of Surgery* 2003 **73** 727–738.
- Wooten MD & King DK. Adrenal cortical carcinoma. *Cancer* 1993 **72** 3145–3155.
- Luton JP, Cerdas S, Billaud L, Thomas G, Guilhaume B, Bertagna X, Laudat MH, Louvel A, Chapus Y, Blondeau P, Bonnin A & Bricaire H. Clinical features of adrenocortical carcinoma, prognostic factors, and the effect of mitotane therapy. *New England Journal of Medicine* 1990 **322** 1195–1201.
- Soreide JA, Braband K & Thoresen SO. Adrenal cortical carcinoma in Norway, 1970–1984. *World Journal of Surgery* 1992 **16** 663–668.
- Copeland PM. The incidentally discovered adrenal mass. *Annals of Internal Medicine* 1983 **98** 940–945.
- Lipsett MB, Hertz R & Ross GT. Clinical and pathophysiologic aspects of adrenocortical carcinoma. *American Journal of Medicine* 1963 **35** 374–383.
- Stratakis CA. Genetics of adrenocortical tumors: gatekeepers, landscapers and conductors in symphony. *Trends in Endocrinology and Metabolism* 2003 **14** 404–410.
- Russell AJ, Sibbald J, Haak H, Keith WN & McNicol AM. Increasing genome instability in adrenocortical carcinoma progression with involvement of chromosomes 3, 9 and X at the adenoma stage. *British Journal of Cancer* 1999 **81** 684–689.
- Beuschlein F, Reincke M, Karl M, Travis WD, Jaurisch-Hancke C, Abdelhamid S, Chrousos GP & Allolio B. Clonal composition of

- human adrenocortical neoplasms. *Cancer Research* 1994 **54** 4927–4932.
- 10 Gicquel C, Leblond-Francillard M, Bertagna X, Louvel A, Chapula Y, Luton J-P, Girard F & Le Bouc Y. Clonal analysis of human adrenocortical carcinomas and secreting adenomas. *Clinical Endocrinology* 1994 **40** 465–477.
 - 11 Kjellman M, Larsson C & Bäckdahl M. Genetic background of adrenocortical tumor development. *World Journal of Surgery* 2001 **25** 948–956.
 - 12 Gicquel C, Raffin-Sanson M-L, Gaston V, Bertagna X, Plouin P-F, Schlumberger M, Louvel A, Luton J-P & Le Bouc Y. Structural and functional abnormalities at 11p15 are associated with the malignant phenotype in sporadic adrenocortical tumors: study on a series of 82 tumors. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 2559–2565.
 - 13 Gicquel C, Bertagna X, Schneid H, Grancillard-Leblond M, Luton J-P, Girard F & Le Bouc Y. Rearrangements at the 11p15 locus and overexpression of insulin-like growth factor-II gene in sporadic adrenocortical tumors. *Journal of Clinical Endocrinology and Metabolism* 1994 **78** 1444–1453.
 - 14 Lack E. Tumors of the adrenal gland and extra-adrenal paraganglia. In *Atlas of Tumor Pathology*, 3rd Series, Fascicle 19, pp 125–151. Ed. J Rosai. Washington, DC: Armed Forces Institute of Pathology, 1997.
 - 15 Weiss LM. Comparative histologic study of 43 metastasizing and nonmetastasizing adrenocortical tumors. *American Journal of Surgery and Pathology* 1984 **8** 163–169.
 - 16 Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J & Speed TP. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Research* 2002 **30** e15.
 - 17 Sturn A, Quackenbush J & Trajanoski Z. Genesis: cluster analysis of microarray data. *Bioinformatics* 2002 **18** 207–208.
 - 18 Higuchi R, Fockler C, Dollinger G & Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* 1993 **11** 1026–1030.
 - 19 Giordano TJ, Thomas DG, Kuick R, Lizyness M, Misek DE, Smith AL, Sanders D, Aljundi RT, Gauger PG, Thompson NW, Taylor JMG & Hanash SM. Distinct transcriptional profiles of adrenocortical tumors uncovered by DNA microarray analysis. *American Journal of Pathology* 2003 **162** 521–531.
 - 20 Yoshida R, Ohuchi N & Kimura N. Clinicopathological study of chromogranin A, B and BRCA1 expression in node-negative breast carcinoma. *Oncology Reports* 2002 **9** 1363–1367.
 - 21 Scopsi L, Sampietro G, Boracchi P & Collini P. Argyrophilia and chromogranin A and B immunostaining in patients with sporadic medullary thyroid carcinoma. A critical appraisal of their prognostic utility. *Journal of Pathology* 1998 **184** 414–419.
 - 22 Yan SF, Fujita T, Lu J, Okada K, Shan ZY, Mackman N, Pinsky DJ & Stern DM. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nature Medicine* 2000 **6** 1355–1361.
 - 23 Gashler A & Sukhatme VP. Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. *Progress in Nucleic Acid Research Molecular Biology* 1995 **50** 191–224.
 - 24 Kartasheva NN, Lenz-Bauer C, Hartmann O, Schäfer H, Eilers M & Döbelstein M. ΔNp73 can modulate the expression of various genes in a p53-independent fashion. *Oncogene* 2003 **22** 8246–8254.
 - 25 Madden SL, Galella EA, Zhu J, Bertelsen AH & Beaudry GA. SAGE transcript profiles for p53-dependent growth regulation. *Oncogene* 1997 **15** 1079–1085.
 - 26 Abdulkadir SA, Qu Z, Garabedian E, Song SK, Peters TJ, Svaren J, Carbone JM, Naughton CK, Catalona WJ, Ackerman JJ, Gordon JI, Humphrey PA & Milbrandt J. Impaired prostate tumorigenesis in Egr1-deficient mice. *Nature Medicine* 2001 **7** 101–107.
 - 27 Adamson ED & Mercola D. Egr1 transcription factor: multiple roles in prostate tumor cell growth and survival. *Tumour Biology* 2002 **23** 93–102.
 - 28 Ji B, Bi Y, Simeone D, Mortensen RM & Logsdon CD. Human pancreatic acinar cells lack functional responses to cholecystokinin and gastrin. *Gastroenterology* 2001 **121** 1380–1390.
 - 29 Ji B, Chen X-Q, Misek DE, Kuick R, Hanash S, Ernst S, Najarian R & Logsdon CD. Pancreatic gene expression during the initiation of acute pancreatitis: identification of EGR-1 as a key regulator. *Physiological Genomics* 2003 **14** 59–72.
 - 30 Bourdeau I, Antonini SR, Lacroix A, Kirschner LS, Matyakhina L, Lorang D, Libutti SK & Stratakis CA. Gene array analysis of macronodular adrenal hyperplasia confirms clinical heterogeneity and identifies several candidate genes as molecular mediators. *Oncogene* 2004 **23** 1575–1585.
 - 31 de Fraipont F, El Atifi M, Cherradi N, Le Moigne G, Defaye G, Houlgatte R, Bertherat J, Bertagna X, Plouin P-F, Baudin E, Berger F, Gicquel C, Chabre O & Feige J-J. Gene expression profiling of human adrenocortical tumors using complementary deoxyribonucleic acid microarrays identifies several candidate genes as markers of malignancy. *Journal of Clinical Endocrinology and Metabolism* 2005 **90** 1819–1829.

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