Identification of Biomarkers of Adrenocortical Carcinoma Using Genomewide Gene Expression Profiling

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**Hypothesis:** The gene expression profiles of benign and malignant adrenocortical tumors are different.

**Design:** Genomewide gene expression profiling and validation.

**Setting:** Tertiary medical center.

**Patients:** Eighty-five patients with benign adrenocortical tumors (n=74) and adrenocortical carcinoma (n=11).

**Intervention:** Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) in 89 adrenocortical tissue samples (11 malignant and 78 benign). The criteria for differentially expressed genes between benign and malignant adrenocortical tumors were a false discovery rate of less than 5% and an adjusted \( P < 0.01 \). Genes differentially expressed by 8-fold higher or lower were validated by RT-PCR.

**Main Outcome Measures:** The diagnostic accuracy of differentially expressed genes as determined by the area under the receiver operating characteristic curve (AUC).

**Results:** We found 37 genes differentially expressed by 8-fold higher or lower. Fifteen genes were downregulated and 22 were upregulated in adrenocortical carcinoma. Of the 37 genes, 29 differentially expressed by microarray correlated with the gene expression levels by quantitative RT-PCR \( (P \leq 0.01) \). Of the 37 genes validated by RT-PCR, 22 were significantly differentially expressed between benign and malignant adrenocortical tumors \( (P < 0.05) \). Five of these 22 genes had an AUC of 0.80 or greater (the AUC for IL13RA2 was 0.90; HTR2B, 0.87; CCNB2, 0.86; RARRES2, 0.86; and SLC16A9, 0.80), indicating high diagnostic accuracy for distinguishing benign from malignant adrenocortical tumors.

**Conclusion:** We identified 37 genes that are dysregulated in adrenocortical carcinoma, and several of the differentially expressed genes have excellent diagnostic accuracy for distinguishing benign from malignant adrenocortical tumors.

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**Adrenocortical Tumors** are common, with a prevalence of approximately 4% in the US population. They are discovered as a result of hormonal hyperscretion (Cushing syndrome, primary hyperaldosteronism, and other conditions), because of local symptoms (back or abdominal pain), and, most frequently, on abdominal imaging for another clinical indication (adrenal incidentaloma). In contrast, adrenocortical carcinoma is rare, with an annual incidence of 2 cases per million persons per year, and it accounts for 0.2% of cancer deaths. Five-year survival for patients with adrenocortical carcinoma varies from 32% to 45%. It is relatively easy to distinguish benign from malignant adrenocortical tumors when there is gross locoregional invasion or metastatic disease. However, most adrenocortical tumors are localized, and there are no clinically reliable criteria to distinguish benign from malignant localized adrenocortical tumors. Consequently, sometimes patients are misdiagnosed as having benign tumors based on histologic examination but later develop aggressive recurrent disease, even after initial complete resection. On the other hand, many patients with adrenal incidentaloma are subjected to adrenalectomy based on the risk of adrenocortical carcinoma as estimated by tumor size, but histologic examination and long-term follow-up show their tumors to be benign. Because of the clinical limitations of reliably distinguishing between benign and malignant adrenocortical tumors, and to gain some biological insight into the pathogenesis of adrenocortical carcinoma, sev-
eral investigators have used genomewide gene expression profiling. Some of these studies7-10 have identified candidate diagnostic biomarkers of adrenocortical carcinoma and molecular profiles that distinguish between benign and malignant adrenocortical tumors. However, the specific candidate genes found in these studies have been discordant, possibly as a result of different approaches to data analysis. In addition, some studies have not validated microarray data using quantitative methods.

We, therefore, analyzed the genomewide expression profile of benign vs malignant adrenocortical tumors to identify candidate markers. We then validated these markers using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) and evaluated their diagnostic accuracy.

## METHODS

### TISSUE SAMPLES

A total of 89 human adrenocortical tissue samples were obtained at surgical resection and were immediately snap frozen and stored at −80°C. Patient and tumor characteristics are summarized in Table 1. Each tumor sample was reviewed again and was confirmed to be adrenocortical tissue. Approval for this study was obtained from the Committee on Human Research at the University of California, San Francisco.

Adrenocortical carcinoma was defined when gross local invasion or lymph or distant metastasis was present at diagnosis or developed during follow-up. The adrenocortical tissue samples were classified as benign if the tumor was localized at presentation and there was no evidence of local or distant recurrent disease after mean follow-up of 2.1 years (range, 1-10 years).

### RNA ISOLATION, PROBE PREPARATION, AND MICROARRAY HYBRIDIZATION

Frozen adrenal tissue was sectioned for RNA isolation, and an adjacent piece was sectioned for routine hematoxylin-eosin staining to confirm tissue diagnosis and type (adrenal cortex and medulla). Total RNA was extracted from homogenized frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, California) and was purified using the RNeasy Mini Kit (Qiagen, Valencia, California). We used 1 µg of total RNA for amplification and labeling with a kit (MessageAmp aRNA Kit; Ambion Inc, Foster City, California). Labeled and fragmented complementary RNA, 12 µg, was hybridized to a gene chip (Affymetrix Human Genome U133 Plus 2.0 GeneChip; Affymetrix Inc, Santa Clara, California) for 16 hours at 45°C. The gene chip arrays were stained and washed (Affymetrix Fluidics Station 400; Affymetrix Inc), according to the manufacturer’s protocol. The probe intensities were measured using an argon laser confocal scanner (GeneArray Scanner; Hewlett-Packard, Palo Alto, California).

### REAL-TIME QUANTITATIVE RT-PCR

Genes differentially expressed in the microarray experiments were validated by means of real-time quantitative TaqMan (Applied Biosystems, Foster City) RT-PCR in individual samples. The same stock of total RNA used for the gene array experiments was used for the real-time quantitative RT-PCR. Total RNA, 125 ng/µL, was reverse transcribed using the RT script complementary DNA synthesis kit (USB Corp, Cleveland, Ohio). Real-time quantitative PCR was used to measure messenger RNA expression levels relative to glyceraldehyde-3-phosphate dehydrogenase messenger RNA expression. The gene expression level is as follows: ΔΔCt = −2 × (Ct of the gene of interest − Ct of glyceraldehyde-3-phosphate dehydrogenase), where Ct is the PCR cycle threshold. The PCR primers and probes for the genes were purchased from Applied Biosystems (Assay-on-Demand Kit). All the PCR experiments were performed in a final volume of 20 µL, with 1 µL of complementary DNA template on a detection system (ABI PRISM 7900 Sequence Detection System; Applied Biosystems). The PCR thermal cycler condition was 95°C for 12 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

### DATA ANALYSIS AND STATISTICAL ANALYSIS

Raw microarray data were analyzed using the Affy package (Affymetrix GeneChip Operating software; Affymetrix Inc) in the free statistical environment R/Bioconductor to generate an intensity value in log2 scale for each probe set using the robust multiarray average method with default variables.11,12 For the class comparison (benign vs malignant), we used the limma package in R/Bioconductor to calculate the moderated t statistic and the associated P values and the log posterior odds ratio (B statistic) that a gene is differentially expressed vs C, not differentially expressed.13 The P values were adjusted for multiple testing by controlling for the false discovery rate using the Benjamini-Hochberg method.14

To evaluate the accuracy of candidate markers to distinguish benign from malignant adrenocortical tumors, we determined the area under the receiver operating characteristic curve (AUC). A stepwise regression analysis was used to determine the AUC for the combination of markers.

### RESULTS

### DIFFERENTIALLY EXPRESSED GENES BETWEEN BENIGN AND MALIGNANT ADRENOCORTICAL TUMORS

Of the 89 adrenocortical tissue samples, 79 (74 cortical adenoma/hyperplasia and 5 malignant) had arrays of ad-
equate quality for data analysis. We performed a class comparison of benign vs malignant adrenocortical carcinoma based on different criteria for differentially expressed genes: (1) fold difference in gene expression levels and (2) a variety of statistical thresholds to account for multiple comparisons. Table 2 summarizes the number of differentially expressed genes based on these criteria. As expected, the number of differentially expressed genes was lower when more stringent statistical criteria were used to account for multiple comparisons.

Thirty-seven genes (15 underexpressed and 22 overexpressed) were differentially expressed by 8-fold higher or lower (range, 8.0- to 24.4-fold change for underexpressed genes and 8.0- to 24.2-fold change for overexpressed genes), with a false discovery rate of less than 5% and an adjusted P < .01. These 37 genes were validated by means of real-time quantitative RT-PCR. Of the 37 genes, 29 showed strong correlation by RT-PCR gene expression levels (P < .05) (Table 3).

**DIAGNOSTIC ACCURACY OF DIFFERENTIALLY EXPRESSED GENES**

By real-time quantitative RT-PCR, the normalized gene expression level of 22 genes was significantly different between benign (54 adrenocortical adenoma, 20 adrenocortical hyperplasia, and 4 normal adrenocortical tissue) and malignant (11 adrenocortical carcinoma) tissue samples (Mann-Whitney test, P < .05). We determined the AUC for 22 of the significantly differentially expressed genes, which ranged from 0.24 to 0.90 (Table 3). Of the 22 genes, 5 (IL13RA2, HTR2B, CCNB2, RARRES2, and SLC16A9) had an AUC of 0.80 or greater, indicating high diagnostic accuracy for distinguishing benign from malignant adrenocortical tumors. The 5 genes in combination showed a slight increase in diagnostic accuracy for distinguishing benign from malignant tumors (Figure). We performed a subset analysis excluding Conn syndrome, because these tumors are rarely malignant, and normal adrenocortical tissue samples. We found that 7 of the 22 differentially expressed genes (IL13RA2, HTR2B, CCNB2, RARRES2, SLC16A9, ALDH1A1, and FREM2) had an AUC of 0.80 or greater, and the combination of the 5 highest AUCs did not show improvement in diagnostic accuracy compared with the highest individual value (AUC, 0.913 for IL13RA2 vs 0.907 for all 5 genes combined). Comparing these markers with tumor size, one of the main current clinical criterion used to assess risk of malignancy demonstrates that the differentially expressed genes are more accurate (Figure).

**COMMENT**

Because there are no reliable markers to distinguish benign from malignant adrenocortical tumors, we studied the genomewide expression profile of adrenocortical tumors to identify candidate diagnostic markers. We validated the microarray gene expression data by using real-time quantitative RT-PCR. The application of stringent filtering criteria yielded 37 candidate diagnostic gene markers significantly differentially expressed between benign and malignant adrenocortical carcinoma. Five of these genes (IL13RA2, HTR2B, CCNB2, RARRES2, and SLC16A9) high diagnostic accuracy (AUC, 0.80).

Of the 5 genes that were the best biomarkers, IL13RA2 had the highest accuracy (overexpressed; AUC, 0.90). IL13RA2 overexpression has been identified in a set of genes that marks and mediates breast cancer metastasis to the lungs.10 HTR2B encodes multiple receptor subtypes of serotonin neurotransmitters. Serotonin is known to act as a growth factor for several types of nontumoral cells and has been proposed to contribute to cell proliferation in aggressive tumors, such as small cell lung, prostate, and colon carcinoma.12 On the other hand, the specific vasoconstrictive effect of serotonin or serotonin receptor agonists might also be useful in inducing hypoxia in tumors, which could be used as a strategy using hypoxia-selective cytotoxins or hypoxia-selective gene therapy.12 CCNB2 is a member of the B-type family of cyclins, which are essential components of the cell-cycle regulatory machinery.13 Cyclin B2 also binds to transforming growth factor β RII; thus, cyclin B2/cdc2 may play a key role in transforming growth factor β-mediated cell-cycle control.14 This gene was also overexpressed in a recent microarray study15 and could be used as a reliable biomarker of lung adenocarcinoma. RARRES2 is a retinoid protein with potent growth inhibitory and cell differentiation activities. IL13RA2 encodes a not well characterized protein involved in cell membrane transportation. The potential implications of these genes in the pathogenesis of adrenocortical carcinoma need to be further investigated.

Frequent chromosomal loci of the most downregulated genes in adrenocortical carcinoma were chromosomes 1, 2, 5, and 7. For upregulated genes, the most common chromosomal loci were chromosomes 2, 8, 9, 12, 15, and 22. These results are in part consistent with those of previous microarray studies.7-10

Among the most differentially expressed genes in adrenocortical carcinoma, we identified GIPC2 (>24-fold change), MGST1 (>22-fold change), IL13RA2 (>24-fold change), and APOBEC3B (>17-fold change), although GIPC2, MGST1, and APOBEC3B were not found to have high diagnostic accuracy. Expression of GIPC2 messenger RNA has been shown to be significantly downregulated in a subset of kidney, colon, and rectal tumors.16 Downregulation of GIPC2 expression in human primary tumors might lead to interference of transforming growth factor β signaling.18 MGST1 is localized in the

<table>
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<th>Statistical Criterion</th>
<th>No. of Genes Differentially Expressed</th>
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<tr>
<td>&gt;2×</td>
<td>1930</td>
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<tr>
<td>Log posterior odds ratio (B statistic) &gt;0</td>
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<tr>
<td>False discovery rate &lt;5%</td>
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<td>Adjusted P&lt;.01</td>
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Table 2. Genes Differentially Expressed Between Benign and Malignant Adrenocortical Tumors by Class Comparison
endoplasmic reticulum and outer mitochondrial membrane, where it is thought to protect these membranes from oxidative stress and to serve as a cellular defense against carcinogens and xenobiotics.20 The APOBEC3B gene encodes a protein that may be an RNA editing enzyme that has roles in growth or cell-cycle control.21 APOBEC3B has also been involved in carcinogenesis of hepatocellular carcinoma through the generation of HBx mutants, providing the hepatocytes with a selective clonal growth advantage.22

We identified several of the most differentially expressed genes that have been previously reported to be dysregulated in adrenocortical carcinoma. This is the case for 2 underexpressed genes: ALDH1A1, an important enzyme of the major oxidative pathway of alcohol metabolism, and RARRES2.9 It is also the case for the overexpressed gene KIAA0101, the regulator of cell proliferation CCNB2, and the regulator of cell division TOP2A.7 The overexpression of TOP2A and the downregulation of HSD3B2 and RARRES2 gene expression have also been documented in a microarray study23 of childhood adrenocortical carcinomas. TOP2A encodes the topoisomerase II α, the direct molecular target of anthracyclines. This gene is frequently coamplified with the HER2 gene in breast cancers, and there is agreement in different studies that TOP2A has a predictive value for the efficacy of

<table>
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<tr>
<th>Gene No.</th>
<th>Gene Symbol</th>
<th>UniGene ID</th>
<th>Spearman Correlation Coefficient for Cct</th>
<th>P Valueb,c</th>
<th>P Valueb,d</th>
<th>AUCc</th>
<th>AUCd</th>
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<tr>
<td>1</td>
<td>ALDH1A1</td>
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<td>&lt;0.01</td>
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<tr>
<td>3</td>
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<td>GIPC2</td>
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<tr>
<td>13</td>
<td>RARRES2</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
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<td>0.01</td>
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<tr>
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<td>SLC16A9</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.80</td>
<td>0.87</td>
</tr>
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</table>

Abbreviations: AUC, area under the receiver operating characteristic curve; Cct, PCR cycle threshold; NA, not applicable; RT-PCR, reverse transcription–polymerase chain reaction.

a The log2 microarray gene expression vs the Cct on RT-PCR was compared using the Spearman correlation coefficient.
b P values were determined by Mann-Whitney test.
c Analysis based on benign (54 adrenocortical adenoma, 20 adrenocortical hyperplasia, and 4 healthy adrenocortical tissue) and malignant (11 adrenocortical carcinoma) tissue samples.
d Subset analysis of benign vs malignant adrenocortical tumors by RT-PCR gene expression, excluding Conn syndrome (n=30) and healthy (n=4) adrenocortical tissue samples.
anthracyclines for the treatment of primary and metastatic breast cancer. Overexpression of the IGF2 gene is one of the most common events seen in adrenocortical carcinoma. We did find several members of the insulin-like growth factor protein family to be upregulated in adrenocortical carcinoma, but their gene expression levels were below the filtering criteria for differential gene expression.

An important consideration when comparing genome-wide gene expression profiling studies in adrenocortical tumors is the type of microarray platform used because results may vary with respect to the number of probe sets, data quality assurance and validation of the microarray data, type of data analysis that accounts for multiple comparisons, and the number of samples analyzed. The focus of this study was to select the most differentially expressed genes because we were interested in identifying candidate diagnostic markers of adrenocortical carcinoma for clinical application. Such an approach obviously may be useful for discovering biomarkers, but it compromises the ability to identify small but significant biological variability in gene expression and differentially involved molecular pathways in adrenocortical carcinoma. Therefore, these issues likely account for the discordances in chromosomal locus and candidate genes between this study and previously published studies.

In conclusion, we identified 37 genes that are dysregulated in adrenocortical carcinoma, and several of these significantly differentially expressed genes have excellent diagnostic accuracy for distinguishing benign from malignant adrenocortical tumors.

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Additional Contributions: Pamela Derish, MA, helped edit this manuscript.

REFERENCES

Michael Bouvet, MD, La Jolla, California: As we have just heard, you have identified 37 genes that are dysregulated in adrenocortical carcinoma, and a number of differentially expressed genes have an excellent diagnostic accuracy for distinguishing benign from malignant adrenocortical tumors. I have several questions for you.

For this information to be useful in determining whether or not to remove a nonfunctioning adrenal mass, a preoperative fine-needle aspiration biopsy would have to be performed. Using your current RNA isolation techniques, would it be feasible to retrieve enough RNA from a needle biopsy to run the microarray analysis? Have you done this yet? Traditionally, we tend to avoid fine-needle aspiration biopsy of adrenal masses. Is there any potential danger of tumor seeding if one biopsies an adrenocortical carcinoma before removing it?

Second, the average size of benign lesions in your series was 3.3 vs 9.9 cm for adrenocortical carcinoma. Your group has previously published in 2006 in the Journal of the American College of Surgeons that size is useful for predicting malignancy and that at a size threshold of greater than or equal to 4 cm, the likelihood of malignancy doubles to 10%, and it is more than 9-fold higher for tumors greater than or equal to 8 cm, where the incidence of malignancy was 47%. The question then becomes, how much more information does microarray analysis really give the surgeon given that size is already an excellent predictor of malignancy? Perhaps patients with intermediate-sized adrenal masses, say 4 to 6 cm, would benefit most from such an approach.

Finally, we are in a new era of targeted therapeutics for many types of cancers. I think that your study does give excellent insight into potential molecular pathways that are involved with adrenocortical carcinoma and opens the door for further studies and may some day have potential therapeutic implications.

Dr Kebebew: I think you have raised 2 important issues with the questions you asked. One is, are we going to be able to translate these markers into clinical practice to make management decisions? I agree with you that routinely we do not do fine-needle aspiration biopsy in our patients with an adrenal incidentaloma to determine if it is malignant. This is because we have not found fine-needle aspiration biopsy to be accurate for distinguishing benign from malignant tumors. You also raised the concern that fine-needle aspiration biopsy could lead to tumor seeding, which is possible but likely to be rare in the borderline cases of adrenal tumor that are 4 to 6 cm in size. As you pointed out, these markers are more likely to be useful for those that are indeterminate, so a large 8- or 10-cm tumor that has suspicious imaging characteristics, such as irregular margins, necrosis, calcification, or high Hounsfield units, should just be resected and not biopsied, and maybe the risk of tumor seeding with fine-needle aspiration biopsy could be a real concern in these tumors. As far as applying the microarray analysis to fine-needle aspiration biopsy samples, some groups have been able to use small amounts of RNA in a variety of tumor samples. Having validated the candidate markers using quantitative real-time RT-PCR, we would be able to use fine-needle aspiration biopsy samples that would yield enough RNA to use. For adrenocortical tumors, we have done this in an ex vivo fashion, and it yields enough RNA material. I should also mention that for the quantitative PCR techniques, which we used to validate the results of the microarray analysis, only a minute amount of RNA is required (50-125 ng of RNA). So, fine-needle aspiration biopsy could be used.

Another situation besides the preoperative evaluation of adrenocortical tumors that these markers may be useful for is in the postoperative diagnosis of distinguishing benign from malignant tumors. We have had several patients who initially underwent complete surgical resection of a 2- to 3-cm adrenocortical tumor that was interpreted as an adrenocortical adenoma on pathology but went on to develop locoregional recurrence as well as distant metastatic disease. So, I think that these markers may be helpful as a postoperative diagnostic adjunct to histologic examination of the resected tumor.

We and others have reported that, yes, tumor size is a useful criterion for predicting the likelihood of malignancy. But, I think we could benefit and our patients could benefit from having more accurate adjunct diagnostic tests to predict which patient is likely to have malignancy or not. Currently, if you consider a patient with a 4-cm adrenocortical tumor, the likelihood is 2-fold higher that the tumor is malignant. Assuming a prevalence of malignancy of 3%, then 10% are malignant postoperatively. That means you would have to resect 9 benign tumors to catch 1 case of adrenocortical carcinoma. Moreover, earlier diagnosis and treatment is the best way that we are going to improve the outcome of patients with adrenocortical carcinoma for which surgical resection is the primary effective treatment modality. Developing adjunct markers to better predict which patient would benefit from early resection at a lower tumor size threshold could benefit our patients.

I do agree that these differentially regulated genes in adrenocortical carcinoma may offer possible targets for therapy; many of these genes have well-characterized functions, some with agents currently available that may be effective at modulating their function.

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