Molecular Classification and Prognostication of Adrenocortical Tumors by Transcriptome Profiling

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Abstract Purpose: Our understanding of adrenocortical carcinoma (ACC) has improved considerably, yet many unanswered questions remain. For instance, can molecular subtypes of ACC be identified? If so, what is their underlying pathogenetic basis and do they possess clinical significance? Experimental Design: We did a whole genome gene expression study of a large cohort of adrenocortical tissues annotated with clinicopathologic data. Using Affymetrix Human Genome U133 Plus 2.0 oligonucleotide arrays, transcriptional profiles were generated for 10 normal adrenal cortices (NC), 22 adrenocortical adenomas (ACA), and 33 ACCs. Results: The overall classification of adrenocortical tumors was recapitulated using principal component analysis of the entire data set. The NC and ACA cohorts showed little intragroup variation, whereas the ACC cohort revealed much greater variation in gene expression. A robust list of 2,875 differentially expressed genes in ACC compared with both NC and ACA was generated and used in functional enrichment analysis to find pathways and attributes of biological significance. Cluster analysis of the ACCs revealed two subtypes that reflected tumor proliferation, as measured by mitotic counts and cell cycle genes. Kaplan-Meier analysis of these ACC clusters showed a significant difference in survival ($P < 0.020$). Multivariate Cox modeling using stage, mitotic rate, and gene expression data as measured by the first principal component for ACC samples showed that gene expression data contains significant independent prognostic information ($P < 0.017$). Conclusions: This study lays the foundation for the molecular classification and prognostication of adrenocortical tumors and also provides a rich source of potential diagnostic and prognostic markers.

Primary tumors of the adrenal cortex are readily classified by routine histopathologic evaluation into benign and malignant groups in the majority of cases using established criteria that include nuclear grade, mitotic rate, presence of atypical mitoses, percent lipid-rich cells, growth pattern, presence of necrosis, and tumor invasion (1). Adrenocortical tumors deemed to lack malignant potential are diagnosed as adrenocortical adenoma (ACA) and represent the majority of these uncommon tumors. In contrast, adrenocortical carcinoma (ACC) possesses, and most often manifests, malignant behavior. These malignant tumors are exceptionally rare with an incidence of one to two cases per million. Adrenocortical tumors are occasionally difficult to classify, and accordingly, are denoted adrenocortical tumors of uncertain malignant potential. Several studies have successfully used immunohistochemistry (IHC) as a supplemental diagnostic tool (2–4). Molecular techniques, such as DNA microarray analysis and evaluation of telomeres, possess significant diagnostic potential (5, 6).

Over the last decade, gene expression profiling via DNA microarray analysis emerged as a useful technique for tumor classification (for examples, see refs. 13–19) and cancer outcome prediction for many solid tumor types (for examples,
and pathologic features of the tissues used are presented in Supplementary Table S1. Mitotic rates were estimated by counting mitotic figures in 50 high power fields (×400).

Single isolates of frozen tissues were used for RNA isolation as previously described (26). Total RNA was isolated from a total of 87 tissues, of which 78 yielded sufficient RNA to be eligible for microarray analysis. Of these, 7 samples were abandoned due to RNA degradation, leaving 71 that were labeled and hybridized to microarrays. After microarray hybridization, six samples were removed from the study set due to poor quality assessment measurements. Fourteen of the remaining 65 samples were repeated from our previous study (21).

cRNA synthesis, oligonucleotide microarray analysis, and processing of microarray data. Preparation of cRNA from total RNA, hybridization, scanning, and image analysis was done according to manufacturer’s protocol and as previously described (26). This study used commercially available high-density oligonucleotide arrays (Human Genome U133 2.0 Plus; Affymetrix) containing 54,675 probe sets. Each probe set usually contains 11 perfectly matched 25-base long probes (PM) as well as 11 mismatch probes that differ by a central base (MM). A representative tumor (ADR016) was selected as the standard and probe-pairs for which the standard had PM-MM of ≤100 were excluded from study (a total of 33,581 probe-pairs). Trimmed-means were computed as described (14), and the standard was scaled to give an average trimmed-mean of 1,000. The data were quantile-normalized and log transformed as described (14). We fit one-way ANOVA models to test for gene differences between ACCs versus ACAs and ACCs versus normal adrenal cortices (NC), and used two-sample t tests to test for differences between subgroups of ACCs. The raw array data (.CEL files) as well as the data set with the statistical tests used to select for differentially expressed genes have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (ref. 27) and are accessible through Gene Expression Omnibus Series accession number GSE10927. The data are supplied as a supplementary excel file, GSE10927_Adrenocortical_logs.xls, in Gene Expression Omnibus but requires ftp downloads from the site ftp-private.ncbi.nih.gov, using the user name georeviewer4, with password GffoX6oB, as do the raw .CEL files. Annotation associating probe sets with Entrez gene identifiers requires ftp downloads from the site ftp-private.ncbi.nih.gov, using the user name georeviewer4, with password GffoX6oB, as do the raw .CEL files. Annotation associating probe sets with Entrez gene identifiers created July 12, 2006, were obtained from the Affymetrix Web site. We did clustering of ACC samples after subtracting the median of each probe set (for ACCs), using average clustering with the usual (Pearson) correlation as the basis for the sample distances (28). Heatmaps were made using Java Treeview (29).

Functional enrichment analysis. We tested groups of probe sets selected as differing between sample groups for overrepresentation of functional gene categories using one-sided Fisher’s exact tests, after first collapsing the lists of probe sets on the arrays to lists of distinct genes with unique Entrez gene identifiers (19,686 distinct genes), and considering a gene as differentially expressed if any probe set for that gene was selected as differing. We tested Gene Ontology biological process terms that were applied to at least 10 genes represented on the arrays (432 such terms), obtained from Affymetrix Web pages. We obtained lists of pathways from the Kyoto Encyclopedia of Genes and Genomes on Aug. 25, 2006 (183 pathways). We obtained version 2 of the Functional Sets (1,687 lists) and Regulatory-motif Sets (837 lists) from the Molecular Signatures Database Web site (30). We tested lists of genes for each chromosome arm derived from Oct. 3, 2006, data from the Human Genome Project.

Quantitative real-time PCR. cDNA was synthesized from 0.5 μg total RNA using a first strand synthesis kit for reverse transcription-PCR (Retroscript; Ambion) and poly(A) primers. The relative abundance of each mRNA species was assessed using real-time quantitative PCR. PCR primers for NOV (NM_002514.3) and NR4A2 (NM_006186.3) were designed using Primer Express software program (ABI) and obtained from Integrated DNA technologies. Glyceraldehyde-3-phosphate dehydrogenase primers were obtained from ABI. Sequences for the primers were NOVprim1 (GCGAGATGGCCAGATG), NOVprim2 (AGGCTCAGGCACTCAG), and NR4A2prim1 (CATATGATCGAGCAGGAA), and

Materials and Methods

**Adrenocortical frozen tissues, histopathology, and RNA isolation.** All of the adrenocortical tissues, except for the eight ACCs obtained from the Cooperative Human Tissue Network (CHTN), were derived from surgical specimens at the University of Michigan Health System (UMHS) and procured by the Tissue Procurement Service of the University of Michigan Comprehensive Cancer Center. Tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura) and stored at -80°C. The UMHS Institutional Review Board approved the laboratory studies.

UMHS-derived tissues were evaluated by a single pathologist (T.J.G.) using standard histopathologic criteria (Weiss criteria; ref. 1) using routine diagnostic H&E stains. Only frozen sections of small tissue pieces were available for CHTN-derived tissues; therefore, the CHTN-contributing diagnoses were accepted without full slide review. Clinical

**Translational Relevance**

Adrenocortical tumors have increasingly risen to clinical attention in recent years because the number of incidentally discovered adrenal masses (so-called adrenal incidentalomas) to assess has steadily increased and the enthusiasm for using targeted treatments for adrenocortical carcinoma (ACC) has rapidly expanded. Here, we used a transcriptomic approach to examine a relatively large cohort of normal adrenocortical tissues and benign and malignant tumors combined with pertinent clinicopathologic data. Using this approach, we showed that gene expression profiles accurately classified these samples and divided the ACCs into two groups that possessed prognostic significance. Furthermore, using multivariate analysis, we showed that gene expression data contained independent prognostic information even when disease stage and mitotic rate were considered. These results should serve as a useful resource for advancing the understanding of adrenocortical tumor pathogenesis and the development of needed diagnostic and prognostic biomarkers.

see refs. 12, 20). A prior DNA microarray study of adrenocortical tumors from our group (21) showed that gene expression profiles can replicate the diagnostic power of morphologic analysis, i.e., separate ACC from ACA, identify low- and high-grade ACCs, and delineate the tumors according to adrenocortical differentiation. However, the small number of samples in this study precluded a class-discovery analysis with the ACCs. Another DNA microarray study used a similar collection of adrenocortical tumors, but with a limited DNA microarray, and identified a putative expression profile with prognostic significance (22). A few other DNA microarray studies have identified genes correlated with tumor diagnosis but were limited by small sample cohorts and/or a lack of clinical data (23, 24). In addition, a recent study focused specifically on pediatric tumors (25). Thus, although these studies have advanced the field, there is a need for an expanded discovery study annotated with clinical data. Here, we profiled gene expression in a relatively large group of normal adrenocortical tissues and benign and malignant tumors with associated clinicopathologic data using a genome-wide DNA microarray.

NR4A2 (GAAGCGCATCTGGCAACTAGA). Quantitative PCR using an ABI7500 was done in duplicate in 30 μl reaction volumes consisting of 1× quantitative PCR SuperMix-UDG SybGreen reaction mix (Invitrogen) supplemented with the appropriate magnesium concentration for the primers used. The reaction conditions were 50°C for 120 s, 95°C for 10 min, followed by 60 cycles of 95°C for 15 s and 60°C for 1 min. The ABI7500 software determined CT automatically. A standard curves for GAPDH was developed from known dilutions of full-length cDNA clones and the CT of NOV and NR4A2 was normalized to glyceraldehyde-3-phosphate dehydrogenase CT of 22.

Adrenal tissue array and cyclin E IHC. An adrenal tissue array, designated Adrenal TMA3, was constructed from paraffin blocks from the UMHS pathology archives and used for a validation study. This array contained a total of 108 adrenocortical tumors (70 ACCs, 24 ACAs) plus 9 macronodular hyperplasia and 5 NCs along with 5 pheochromocytomas and 16 miscellaneous normal controls arrayed in single 1.0 mm diameter cores. Five of the 24 ACAs (20.8%) and 16 of the 70 ACCs (22.8%) contained in this array were also used for DNA microarray analysis.

IHC for cyclin E was done using Adrenal TMA3 and a mouse monoclonal anti-cyclin E antibody (clone HE12; catalog 32-1600; Invitrogen Corp.) at 1:400 dilution. IHC was done using a Dako autostainer with citrate buffer pretreatment (pH 6). Primary antibody was detected using the Dako LSAB Plus kit (Dako Corp.). Cyclin E immunoreactivity was scored by assessing the percentage of positive tumor cells. Results were grouped into the following categories: <5%, 1+: 5% to 50%, 2+: >50%, 3+.

Clinical annotation. The University of Michigan Endocrine Bank contains the Endocrine Database, which securely joins clinical data about a patient’s course of disease, pathology data, and outcome of treatment. The database is Institutional Review Board approved as a repository of retrospectively gathered patient data behind the UMHS firewall and under control of a small research team. Each research project that intends to query these data to address a research hypothesis undergoes a separate Institutional Review Board approval process based on its own merits and risks, as did this study.

Survival analysis. In survival analysis, we right truncated survival data at 5 y. Mitotic rates, m, were log-transformed using log(max(m,0.5)). The first principal component for just ACC samples was standardized by subtracting the mean and dividing by the SD (which was 15.6 units). We treated the 2 metastatic tumor samples as stage 4 samples in the survival analysis.

Results

Gene expression profiles replicate the overall morphologic classification of adrenocortical tumors. Gene expression profiles of 65 adrenocortical tissues (10 NCs, 22 ACAs, and 33 ACCs) were generated using oligonucleotide arrays with 54,675 probe sets representing ~20,000 genes. We used principal components analysis (PCA) with data from all probe sets to exhibit the two-dimensional views that contain the greatest amount of variability in the data. The resulting PCA view recapitulated the overall morphologic classification of the three groups of samples (Fig. 1). The ACC cohort was clearly separated from the ACA and NC cohorts, indicating that there were many gene expression differences between the ACC and ACA and between ACC and NC. Low-grade ACCs tended to be located closer to the ACA samples than high-grade ACCs (Fig. 1).

With regard to variability within diagnostically similar tissues, the least amount of variability was seen in NC cohort. This was an expected result as normal tissues generally show less gene expression variation compared with tumors, despite the fact that four NCs contained some contaminating normal
adrenal medulla as assessed by elevated levels of tyrosine hydroxylase transcripts (TH, 208291_s_at). None of the ACA or ACC samples exhibited elevated TH levels, indicating these samples are free of medullary contamination.

The ACA group showed an intermediate degree of variability in the PCA view (Fig. 1). This group was immediately adjacent to the NC group and occupied a location that was between the NCs and ACCs. These observations are consistent with the histopathology of ACA, specifically that ACAs more closely resemble NC than ACC.

The ACC group showed a much larger degree of variability relative to the other two groups (Fig. 1). This observation is also consistent with the histopathology of ACC, which exhibits a range of tumor morphologies. Collectively, these results are similar to and extend those produced with a smaller tumor cohort and a more limited DNA microarray (21).

One ACC, given its histopathology, was located in a remarkable location in the PCA view (Fig. 1). ACC053 was one of the tumors closer to the ACA cohort, a result consistent with its pathology and exceptionally low mitotic rate of 1 mitotic figure per 50 high-powered fields. This tumor, derived from the CHTN and diagnosed as ACC at the CHTN-contributing institution (see Materials and Methods), was notably large (19.0 cm, 2,310 grams), yet lacked the usual morphologic features characteristic of malignant behavior (e.g., capsular and vascular invasion). The determination of malignancy at the CHTN-contributing institution was based entirely on tumor size. We therefore suggest, based on our analysis of its gene expression profile (Fig. 2), that ACC053 may be a giant ACA rather than an ACC (31). Clinical follow-up revealed no evidence of recurrent or metastatic disease 2 years after resection and the patient died of other causes.

Identification of differentially expressed transcripts. One of the primary goals of this study was to identify those transcripts that are preferentially present or absent in ACC compared with NC and ACA. We selected probe sets for which the P values for comparing ACC versus ACA and ACC versus NC were both <0.001, and for which the average fold-differences both indicated at least 1.5-fold increases or 1.5-fold decreases for the two comparisons. These criteria selected 2,875 probe sets, 1,300 as increased in ACCs, and 1,575 with lower expression in ACCs (879 and 1,011 distinct genes, respectively, 1,890 total). We estimated the number of these selected probe sets likely to be false-positive findings by performing an identical analysis of 1,000 data sets in which the sample labels were randomly permuted, and obtained only 0.10 qualifying probe sets on average, indicating that our selected gene list is of exceptionally high quality. Supplementary Table S2 gives more detailed results for this as well as some other comparisons. A supplementary table (deposited in Gene Expression Omnibus) indicates the complete list of selected probe sets, of which a very small subset is shown in Fig. 2. This gene list contains many genes related to adrenocortical tumors, including IGF2, SPP1, TOP2A, ENC1, and H19.

Functional enrichment analysis of the ACC profile. We then analyzed the list of 1,890 differentially expressed genes to...
determine if these genes are overrepresented in other biologically relevant lists. We chose several lists for comparison, such as Gene Ontology terms, Kyoto Encyclopedia of Genes and Genomes pathway terms, Molecular Signatures Database, miRNA target gene lists, and lists of genes assigned to specific chromosome arms. Key results are presented in Supplementary Table S3. Many of the most highly significant results are related to cell proliferation, an expected finding given the high mitotic rate commonly observed in ACCs. The chromosome arm enrichment data, suggesting increased copy numbers of 12q and 5q and decreased copy numbers of 11p, 1p, and 17p, is in good agreement with previous studies using CGH (32–34).

We tested a list of genes correlated with measures of chromosomal instability and “functional aneuploidy” from mRNA array data (35), after noticing that many of the most significantly increased transcripts in ACCs were on this list. Of the 70 genes listed, 48 were selected as increased in ACCs in our data, where only 3.1 would be expected by chance ($P = 1.5 \times 10^{-48}$) and no genes on the list were selected as decreased in ACC.

**Evidence for perturbation of the IGF2 locus in ACC.** Perturbation of the IGF2 locus at 11p15.5 is one of the most consistent and dominant genetic changes in ACC (for reviews, see refs. 36, 37). In this study, 28 of 33 ACCs (84.8%) showed markedly increased levels of IGF2 transcripts as measured by two probes sets (202410_s_at and 210881_s_at) compared with NC and ACA (Fig. 2). The H19 gene shows a near-reciprocal pattern of expression (Fig. 2), consistent with the observed altered methylation status of the H19 promoter in ACC (38).

**Quantitative reverse transcription-PCR and IHC validation studies.** Quantitative reverse transcription-PCR was done using the same RNA preparations used for microarray analysis for two differentially expressed genes, NOV and NR4A2. The mean CT values NC, ACA, and ACC for NOV were 20.5, 19.1, and 22.3, respectively, and for NR4A2 were 24.0, 23.5, and 27.1, respectively. The results confirm the relative decreased expression of these genes in ACC compared with NC and ACA.

IHC was also done to provide validation of the microarray data. Cyclin E was chosen as an immunohistochemical antigen for several reasons: its expression has been reported to be increased in ACCs compared with ACAs (39), it was one of the differentially expressed genes identified in our analysis, and the availability of anticyclin E antibodies that robustly detect cyclin E antigen in formalin-fixed, paraffin-embedded tissues. Cyclin E IHC, done using Adrenal TMA3, which is predominantly independent of the cases used for DNA microarray analysis, showed increased cyclin E protein expression in the ACC cohort compared with the NC and ACA cohorts (2-fold increase in ACC versus ACA, $P < 0.0001$; Supplementary Fig. S1). This result was consistent with the DNA microarray data for the 3 cyclin E probe sets present on the array (CCNE1, 213523_s_at; CCNE2, 205034_s_at and 205034_s_at), thereby providing additional validation of the microarray data.

**Subclassification of the carcinomas reveals two groups related to tumor grade and proliferation, chromosomal regions of expression, and functional aneuploidy.** We then did hierarchical clustering using “average” clustering of the 33 ACC samples using data from all probe sets in an attempt to perform class discovery within the ACC group. The resulting dendrogram divided the samples into two nearly equally sized clusters, designated ACC Cluster 1 and Cluster 2 (Fig. 3). We also computed the principal components for just the 33 ACA samples, using all probe sets on the array, and not surprisingly found that the clusters approximately divided the samples.
along the first principal component (Fig. 1). A strong
relationship was observed between these clusters and tumor
grade (i.e., mitotic rate measured by mitotic counts; \( P = 0.004 \),
two-sided Fisher’s exact test; Fig. 1). ACC Cluster 1 consisted
predominantly of high-grade tumors (14 of 16, 87.5%),
whereas ACC Cluster 2 contained predominantly low-grade
tumors (11 of 17, 64.7%). Thus, cluster designation imperfectly
reflects tumor mitotic grade.

Using two-sample \( t \) tests comparing ACC Cluster 1 and 2, we
asked that probe sets give \( P \) values of <0.001 and fold
differences of at least 1.5-fold, giving 1,241 increased and 872
decreased probe sets in ACC Cluster 1, which collapsed to 829
increased and 626 decreased distinct genes. We did enrichment
analysis using these genes, and as shown in Supplementary
Table S4, again identified proliferation-related gene sets as
being preferentially present in ACC Cluster 1. The 70 genes
associated with functional aneuploidy (35) were overrepresent-
ed in genes found increased in ACC Cluster 1. Also, the
intersection of genes found increased in ACC that were also
increased in ACC Cluster 1 versus ACC Cluster 2 was highly
significant. These results suggest that as a generalization, ACC
Cluster 1 samples consist of more extreme tumors, differing
from benign tumors to a greater extent than samples in ACC
Cluster 2. The regional chromosomal differences in expression,
particular those found for 1q, 16p, and 5q, do not however
paint a similar picture; the first two having not been very
significant in the comparison of ACC and other samples,
wheras 5q genes were significantly increased in ACC samples
but are here found decreased in ACC Cluster 1 samples.

Survival analysis of gene expression and clinicopathologic
variables. Using 24 patients from UMHS with follow-up data,
we did Kaplan-Meier analysis and found that patients in ACC
Cluster 1 had poorer survival than patients in ACC Cluster 2
(\( P = 0.020 \), log-rank test; Fig. 4A). Patients with high-grade
tumors had worse outcomes but the results were not significant
(\( P = 0.09 \); Fig. 4B). However, when we used a more fine-grained
measure of grade, by log-transforming the mitotic rates, this
was significantly associated with survival using Cox propor-
tional hazards models (\( P = 0.027 \), Wald test). Similarly, we
standardized the first principal component from the array data,
which is a more detailed score than simply using the cluster,
and obtained a more significant result than using just cluster
(\( P = 0.006 \)). Because we had very few stage 1 or 3 patients, we
grouped stages 1 and 2, and stages 3 and 4. We then fit a
multivariate Cox model with 2 combined stage groups, the
transformed mitotic rate, and the first principal component. As
shown in Table 1A, the first principal component remained
significant (\( P = 0.017 \)), indicating that it contains significant
prognostic information beyond that contained in the stage and
mitotic rate data. Stage 3 and 4 patients had worse outcomes
than stage 1 and 2 patients, as expected (\( P = 0.014 \); Fig. 4C).
When we tested the 10 probe sets most correlated with the first
principal components (after summing their standardized values
and then standardized this sum), we obtained approximately
the same result (Table 1B). This, however, is expected because
this TenGeneScore was highly correlated to the first principal
component (\( r = 0.986 \)) and it is likely that the next most
correlated 10 genes would perform similarly to the ones we
actually used.

**Discussion**

ACC, compared with most other carcinomas, is an extremely
rare disease and accordingly challenging to study. The
unavailability of large series of cases precludes many types of
analyses, especially those designed to identify clinicopathologic
variables related to treatment and survival, although progress
related to the efficacy of mitotane therapy has been recently reported (40). The need for such studies is great and will continue to grow as novel targeted therapies are used to treat patients with ACC.

Our DNA microarray analysis clearly shows the power of molecular profiling as a tool for the diagnosis of adrenocortical tumors. Microarray-based assessment can accurately separate ACAs from ACCs and may actually do so with slightly higher accuracy than morphology, given that one tumor diagnosed as an ACA may be a large ACA. Use of DNA microarrays as an adjunctive diagnostic tool may be useful, especially at centers with limited experience with these rare tumors. Additionally, we provide a rich source of potential diagnostic markers that could be developed into useful immunohistochemical tools, to be used singly or in small panels. These genes, some of which are shown in Fig. 1, includes many cell cycle and proliferation genes (e.g., CCNB2, ASPM, RRM2, TOP2A, and CDKN3), as well as genes known to play a role in tumor invasion in other carcinoma types (e.g., SPP1).

The main significance of this study lies in the integration of a large cohort of normal tissues and benign and malignant tumors with associated clinicopathologic and genome-wide transcriptional profiles. Our previous work in this area (21) was limited by the smaller number of samples and lack of outcome data and, thus, several important questions were not addressed. For example, it could not be determined whether ACC can be divided into clinically relevant subtypes based on expression profiles and whether gene expression information provides useful information beyond that provided by standard clinicopathologic analysis. Other DNA microarray work used a similar series of tumors but with a limited DNA microarray (22). The present study overcomes these shortcomings.

Our results, together with what is known about the significance of mitotic rate grading (3, 7–11), strongly confirm the important role of cell growth and proliferation as a prognostic factor for ACC. However, the most novel aspect of our study is the finding that gene expression data contains independent prognostic information even when mitotic rate and stage data are included in the multivariate analysis. Thus, our study indicates that it should be possible to provide a more refined prognostic evaluation of ACCs based on gene expression. Future efforts will be directed at distilling this result into a manageable assay that can be used using routinely fixed ACC tissues. Our results using a 10-gene panel of prognostic genes suggest that this is feasible. In the meantime, pathologists should consider routinely reporting actual mitotic rate counts rather than simple low- or high-grade assessment.

It is possible using other array data (with stage and mitotic rate estimates) to obtain the value of each tumor for our principal component 1, which is just a linear combination of the values from the array, and so to obtain the estimate of the relative risk for each patient using our Cox model. However, our small sample size and the lack of an independent data set on which to test the predictions of our fitted Cox model dictate caution in recommending such a procedure. Data on more patient samples will likely lead to improved risk prediction, or better indicate which particular pathway alterations or genetic mutations are primarily responsible for the predictive ability of the gene expression values.

The high degree of overlap in gene expression between ACC Cluster 1 and a set of genes associated with chromosomal instability and tumor aneuploidy (35) is entirely consistent with the idea that adrenal cancer follows the common cancer paradigm in which genomic instability leads to gross chromosomal changes and aneuploidy. The available cytogenetic data on adrenal tumors (32–34, 41, 42) is also consistent with this model.

Enrichment analysis of the expression data predicted possible gains of 12q and 5q and possible loss of 11q, 1p, and 17p in ACC. A similar analysis of the ACC clusters predicted possible gains of 1q, 22q, 6q, 10p, and 6p in Cluster 1 ACCs. These findings suggest that these regions represent deletions of tumor suppressor genes or amplifications of oncogenes. Comparison with the available CGH data (32–34, 41, 42) shows a high degree of agreement for some changes, suggesting that expression data could be combined with array CGH data to pinpoint the specific causative amplifications and deletions within these large chromosomal regions.

Increased **IGF2** expression was identified in this study as one of the most dominant transcriptional changes specifically present in ACC relative to ACA and NC, as it was in our prior microarray study (21). This finding is consistent with a large

### Table 1. Multivariate survival analysis

<table>
<thead>
<tr>
<th>Effect</th>
<th>Coefficient</th>
<th>SE</th>
<th>P (Wald test)</th>
<th>Relative risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 3-4 vs 1-2</td>
<td>1.39</td>
<td>0.567</td>
<td>0.014</td>
<td>4.03 (1.33-12.24)</td>
</tr>
<tr>
<td>Principal component 1</td>
<td>1.00</td>
<td>0.421</td>
<td>0.017</td>
<td>2.73 (1.19-6.23)</td>
</tr>
<tr>
<td>Log (mitotic rate)</td>
<td>1.55</td>
<td>0.940</td>
<td>0.100</td>
<td>4.70 (0.74-29.66)</td>
</tr>
<tr>
<td>B. Multivariate Cox proportional hazard model for patient survival, using stage (1-2 vs 3-4), TenGeneScore for the ACC samples, and base-2 log-transformed mitotic rates</td>
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<tr>
<td>Stage 3-4 vs 1-2</td>
<td>1.47</td>
<td>0.574</td>
<td>0.011</td>
<td>4.34 (1.41-12.35)</td>
</tr>
<tr>
<td>TenGeneScore</td>
<td>0.94</td>
<td>0.409</td>
<td>0.021</td>
<td>2.57 (1.15-5.73)</td>
</tr>
<tr>
<td>Log (mitotic rate)</td>
<td>1.54</td>
<td>0.916</td>
<td>0.094</td>
<td>4.64 (0.77-27.92)</td>
</tr>
</tbody>
</table>

NOTE: The top 10 genes correlated to principal component 1 were **CSTA**, **RALA**, **VACT14**, **APOOL**, **MOSPD1**, **PRLD3**, **TFE3**, **PRR3**, **C5orf32**, and **KIF5B**. Abbreviation: CI, confidence interval.
body of published literature on perturbation of the IGF2 locus in ACC (for reviews, see refs. 36, 37, 43–45). Although the molecular basis for the 2-fold elevated level of IGF2 transcription in familial ACC associated with BWS is pathologic imprinting of the IGF2/H19 locus and paternal isodisomy, the markedly elevated IGF2 expression (with concomitant down-regulation of H19) in sporadic ACC is likely to involve additional mechanisms of transcriptional regulation (43). Regardless of the precise mechanisms leading to increased expression, IGF2 has a mitogenic effect and is directly involved in the proliferation of the adrenal cancer cell line NCi H295R via an IGFR-dependent mechanism (46). This autocrine stimulatory loop, together with the IGF2 expression pattern in adrenocortical tumors (90% of ACCs and rare in ACA), makes targeting the IGF system an attractive therapeutic approach for ACC (47–49). Accordingly, multi-institutional trials with an anti-IGFIR monoclonal antibody are being developed.

Enrichment analysis of the ACC genes identified a significant number of genes containing the binding domain for the E2F transcription factor. This is consistent with a bioinformatic study that revealed up-regulation of E2F-regulated genes as a common event across a broad range of tumor types, including ACC (50).

We fully expect that our diagnostic and prognostic results will be broadly applicable to adult ACC. Although our ACC cohort does include 2 pediatric cases that were typical of the other ACCs, this number is too small to make a valid assessment about differences between pediatric and adult ACCs. We also fully expect that our data can be used to classify individual adrenocortical tumors, either by performing DNA microarray analysis or by a multiplex quantitative reverse transcription-PCR approach using a selected set of informative genes combined with a simple statistical classifier such as a nearest neighbor classifier. Furthermore, it should also be possible to use our data to develop novel IHC markers into useful diagnostic and prognostic markers. Future efforts will focus on translating our results into clinically useful tools for the pathologic evaluation of these tumors.

In summary, DNA microarray analysis of a large group of adrenocortical tumors accurately classified benign and malignant tumors, confirmed the diagnostic and prognostic importance of cell growth and proliferation in ACC, and divided the malignant tumors into two groups that possessed prognostic significance. In addition, gene expression profiles provided prognostic information independent of tumor mitotic rate and stage. Looking forward, if effective targeted therapies for ACC are developed in the not too distant future, then the results of this study strongly suggest that it will be possible and desirable to use DNA microarray analysis, or a defined panel of genes, to simultaneously confirm the diagnosis of ACC, determine a more precise prognosis, and assist in the selection of appropriate therapy. Although expensive and technically challenging, a microarray-based assay that delivers such relevant information would significantly advance the care of ACC patients and represent a large step toward the realization of personalized genomic medicine for these patients.

Disclosure of Potential Conflicts of Interest

T.J. Giordano, shareholder, Affymetrix, Inc. No other conflicts are disclosed.

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