Abstract

Purpose: Adrenocortical adenomas are common, whereas adrenocortical carcinomas are rare. Discriminating between benign and malignant adrenocortical tumors using conventional histology can be difficult. In addition, adrenocortical carcinomas generally have poor prognosis and limited treatment options. MicroRNAs are short noncoding RNAs that are involved in regulation of gene transcription.

Experimental Design: To identify microRNAs involved in the pathogenesis of adrenocortical tumors, expression profiling of microRNAs was done on a cohort of 22 adrenocortical carcinomas, 27 adrenocortical adenomas, and 6 normal adrenal cortices.

Results: Twenty-three microRNAs were found to be significantly differentially expressed between adrenocortical carcinomas and adrenocortical adenomas. miR-335 and miR-195 were significantly downregulated in adrenocortical carcinomas compared with adrenocortical adenomas. This result was further validated in an external cohort of six adrenocortical carcinomas and four adrenocortical adenomas. Using Kaplan-Meier analysis, downregulation of miR-195 and upregulation of miR-483-5p in adrenocortical carcinomas were significantly associated with poorer disease-specific survival.

Conclusions: These findings indicate that deregulation of microRNAs is a recurring event in human adrenocortical carcinomas and that aberrant expression of miR-195 and miR-483-5p identifies a subset of poorer prognosis adrenocortical carcinomas.

Adrenal tumors are common, occurring in 3% of patients older than the age of 50 years. Adrenocortical carcinomas, however, are rare, with a prevalence of 4 to 12 per million population (1). Discriminating between benign and malignant adrenocortical tumors using conventional histology can be difficult. In addition, adrenocortical carcinomas generally have poor prognosis and limited treatment options (2, 3). Identification of molecular markers that can accurately differentiate adrenocortical carcinomas from adrenocortical adenomas and that can serve as prognostic markers, as well as therapeutic targets, for adrenocortical carcinomas would be of great value in clinical management.

In the past year, three large series have shown that microarray gene expression profiling of adrenocortical carcinomas correctly assigns adrenocortical tumors as benign or malignant (4–6). Furthermore, two of the studies were able to divide adrenocortical carcinomas into low- and high-grade groups with different survival and outcome (4, 5).

MicroRNAs are noncoding, 20- to 22-nucleotide RNAs that negatively regulate gene expression in a sequence-specific manner and have wide ranging roles in development, differentiation, growth, and apoptosis. They interact with target mRNAs at specific sites to induce cleavage of the message or inhibit translation. MicroRNAs therefore may reduce the protein levels of their target genes without altering the mRNA levels (7). In animals, microRNAs are transcribed from intergenic or intronic DNA as large precursors, termed pri-microRNAs, which then undergo successive enzymatic processing by Drosha, Pasha, and Dicer into their mature, 22-nucleotide RNA, termed the miRNA:mRNA* duplex. This duplex is incorporated into the RNA-induced silencing complex, and the mature microRNA strand is preferentially retained (8).
Translational Relevance
In this study, using microarray profiling, we identified that miR-335 and miR-195 were significantly downregulated in adrenocortical carcinomas compared with adrenocortical adenomas. To our knowledge, this is the first report of its kind. This result was further validated in an external cohort of six adrenocortical carcinomas and four adrenocortical adenomas. Using Kaplan-Meier analysis, downregulation of miR-195 and upregulation of miR-483-5p in adrenocortical carcinomas were significantly associated with poorer disease-specific survival. With further studies, miR-195 and miR-483-5p may prove to be useful in predicting a subset of patients with adrenocortical carcinomas with poor outcome, who may benefit from the increasing number of adjuvant therapeutic options being tested in adrenocortical carcinoma. Indeed, miR-195 and miR-483-5p may be proven in time to be therapeutic targets themselves, as seen with miR-26 in a hepatocellular cancer mouse model.

There is increasing recognition that microRNAs are involved in carcinogenesis. Such microRNAs, termed oncomirs, act as tumor suppressor or oncogenes (7, 9). A number of malignancies have been associated with characteristic microRNA signatures (10, 11). MicroRNAs have been found to be dysregulated in cancers compared with normal tissue, such as miR-143 and miR-145, which have been found to be downregulated in colorectal (12) and breast cancer (13), and miR-21, which have been found to be upregulated in glioblastomas (14, 15) and breast cancers (13).

To date, there has been no large study examining the role of microRNAs in the pathogenesis of human adrenocortical cancer. We have therefore investigated the microRNA profiles of 21 adrenocortical carcinoma patients (22 adrenocortical carcinoma samples) with long-term clinical follow-up and compared their profiles with 27 adrenocortical adenomas and six normal adrenal tissues. Results were validated in an external cohort of six adrenocortical carcinomas and four adrenocortical adenomas.

We report a characteristic microRNA signature in human adrenocortical carcinomas, whereby miR-335 and miR-195 are significantly underegulated and miR-483 is overexpressed in adrenocortical carcinomas compared with adrenocortical adenomas. Most importantly, differential expression of miR-195 and miR-483-5p identified a group of poorer prognosis adrenocortical carcinomas.

Materials and Methods
This study used RNA from six normal adrenal cortices, 27 adrenocortical adenomas, and 22 adrenocortical carcinomas. The samples were hybridized to Exiqon miCURY LNA Arrays version 10.0, which contains ≥1,000 capture probes targeting all human, mouse, and rat microRNA sequences annotated in miRBase 10.0 and also covers 847 mature microRNAs in miRBase version 11.0.

Patients and tumors. Ethics approval for the study was obtained from the Northern Sydney Area Health Service Human Research Ethics Committee. Ethics approval was also obtained from the University of California Los Angeles Institutional Review Board. Informed consent was obtained from patients before sample collection. Viable tumor tissue was surgically removed and snap frozen in liquid nitrogen. The samples were stored at -80°C in the Neuroendocrine Tumor Bank of the Kolling Institute of Medical Research, which contains tumors collected from 1992 to the present. The tumors were reviewed by a single pathologist (A.J. Gill), who was blinded to other data. Tumors with a Weiss score of ≤3 were classified as adrenocortical adenomas, whereas those with a Weiss score of ≥4 were considered to be adrenocortical carcinomas (16, 17). Patients with adrenal tumors as part of a known familial syndrome were excluded on clinical grounds. Six normal adrenal cortex samples were used for the microarray study. Normal adrenal cortex was obtained from adenectomy samples away from the site of the tumor.

Survival in months was calculated from the date of the first operation to date of death or censor date of March 31, 2009. Survival data was available for 18 of the 21 patients with adrenocortical carcinoma.

RNA extraction. Total RNA was extracted from fresh frozen tissue, as previously described (18). At the time that tumor samples were cut for RNA extraction, representative portions of the frozen tissue were extracted and then fixed and embedded in paraffin for histologic assessment. A single observer (A.J. Gill), blinded about other data, confirmed that the tissue consisted of either adrenocortical adenoma or adrenocortical carcinoma and then calculated the ratio of tumor cells to nonneoplastic cells (e.g., lymphocytes, endothelial cells, or nonneoplastic adrenal) in the processed tissue based on morphology. Only samples that were shown on histology to contain at least 80% tumor cells were included in this study. The normal adrenal cortex samples were obtained from the adrenal cortex of adrenocystectomy samples away from the site of the adrena.

The quality of total RNA was verified with the Agilent 2100 Bioanalyzer. Microarray preparation. The discovery cohort used in microarray analysis consisted of 22 adrenocortical carcinomas, 27 adrenocortical adenomas, and six normal adrenal samples from the Neuroendocrine Tumor Bank at the Kolling Institute. MicroRNA microarray profiling was done by Exiqon, Denmark using miCURY LNA array version 10.0 (Exiqon). Briefly, 1 μg total RNA from sample and reference were labeled with Hy3 and Hy5 fluorescent label, respectively, using the miCURY LNA Array power labeling kit (Exiqon), as described by the manufacturer. The Hy3-labeled samples and an Hy5-labeled reference RNA sample were mixed pairwise and hybridized to the miCURY LNA array. The hybridization was done according to the miCURY LNA array manual using a Tecan HS4800 hybridization station (Tecan). After hybridization, the microarray slides were scanned and stored in an oxygen-free environment (ozone level, <2.0 ppb) to prevent potential bleaching of the fluorescent dyes. The miCURY LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc.), and the image analysis was carried out using the ImaGene 7.0 software (BioDiscovery, Inc.). The quantified signals were background corrected using the “Normexp with offset value 10” (19) followed by normalization using the global Lowess (locally weighted scatterplot smoothing) regression algorithm.

Quantitative reverse transcriptase-PCR. Key microarray microRNAs were validated using quantitative reverse transcriptase-PCR on a combination of samples that consisted of a randomly selected subgroup of the original discovery cohort together with an independent cohort of tumors from the Kolling Institute and the University of California Los Angeles. Therefore, from the original discovery cohort, four (of 27) adrenocortical adenomas and four (of 22) adrenocortical carcinomas and an additional five adrenocortical adenomas (from the Kolling Institute) and six adrenocortical carcinomas (from UCLA) were included. Quantitative reverse transcriptase-PCR was used to quantify microRNA levels of miR-195 (TaqMan MicroRNA Assay 4323105), miR-335 (4373045), miR-483 (4378094), and miR-7 (4373014). For miR-195, miR-335, and miR-483, the gene expression pattern of these tumors was compared...
with a reference sample that consisted of a pooled sample of four normal adrenal cortices. For miR-7, which had been identified as differentially expressed between adrenocortical neoplasia (either adrenocortical carcinoma or adrenocortical adenoma) and normal, the reference sample used in quantitative reverse transcriptase-PCR was a pooled sample of one adrenocortical carcinoma, one adrenocortical adenoma, and one normal. Quantitative reverse transcriptase-PCRs were done in triplicate in two independent experiments. Total RNA (5 ng for miR-195, miR-335, and miR-7; 50 ng for miR-483) was converted to cDNA using primers specific for each microRNA and the TaqMan MicroRNA reverse transcription kit (4366597; Applied Biosystems), according to the manufacturer's instructions. All quantitative reverse transcriptase-PCRs were done using a 5′ nucleate technique with specific TaqMan Gene Expression Assays (Applied Biosystems) and Taqman Universal PCR Master Mix, NO AmpErase UNG (Applied Biosystems), on a Rotorgene 3000 (Corbett Research). RNU48 (4373383) was used as the endogenous control for normalization.

### Table 1. Clinical characteristics of microarray patients

<table>
<thead>
<tr>
<th></th>
<th>Adrenocortical carcinomas</th>
<th>Adrenocortical adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>No. of samples</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Primary tumor</td>
<td>19</td>
<td>27</td>
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<tr>
<td>Metastasis</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>45 ± 17</td>
<td>54 ± 14</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>84.8 ± 32.2</td>
<td>30.3 ± 11.4</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>385.9 ± 385.4</td>
<td>27.4 ± 16.6</td>
</tr>
<tr>
<td>Weiss score</td>
<td>5.5 ± 2.4</td>
<td>0.4 ± 0.7</td>
</tr>
</tbody>
</table>

### Table 2. List of significantly differentially expressed microRNAs between adrenocortical carcinomas and adrenocortical adenomas

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Upregulated in ACCs compared with ACAs</th>
<th>P</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-339-5p</td>
<td></td>
<td>4.05 × 10^{-6}</td>
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</tr>
<tr>
<td>hsa-miR-130b</td>
<td></td>
<td>8.28 × 10^{-8}</td>
<td>+1.5</td>
</tr>
<tr>
<td>hsa-miR-483-5p</td>
<td></td>
<td>1.29 × 10^{-7}</td>
<td>+4.1</td>
</tr>
<tr>
<td>hsa-miR-106b</td>
<td></td>
<td>1.47 × 10^{-6}</td>
<td>+1.6</td>
</tr>
<tr>
<td>hsa-miR-148b</td>
<td></td>
<td>4.36 × 10^{-6}</td>
<td>+1.5</td>
</tr>
<tr>
<td>hsa-miR-93</td>
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<td>7.18 × 10^{-6}</td>
<td>+1.7</td>
</tr>
<tr>
<td>hsa-miR-135a</td>
<td></td>
<td>1.71 × 10^{-5}</td>
<td>+2.3</td>
</tr>
<tr>
<td>hsa-miR-320a</td>
<td></td>
<td>1.83 × 10^{-5}</td>
<td>+1.5</td>
</tr>
<tr>
<td>hsa-miR-503</td>
<td></td>
<td>2.15 × 10^{-5}</td>
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</tr>
<tr>
<td>hsa-miR-450a</td>
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<td>2.36 × 10^{-5}</td>
<td>+2.1</td>
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<td>hsa-miR-542-3p</td>
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<td>2.40 × 10^{-5}</td>
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<td>hsa-miR-143</td>
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<td>hsa-miR-542-5p</td>
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<td>5.86 × 10^{-5}</td>
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<table>
<thead>
<tr>
<th>miRNA</th>
<th>Downregulated in ACCs compared with ACAs</th>
<th>P</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-335</td>
<td></td>
<td>8.63 × 10^{-11}</td>
<td>-4.0</td>
</tr>
<tr>
<td>hsa-miR-195</td>
<td></td>
<td>1.97 × 10^{-8}</td>
<td>-2.8</td>
</tr>
<tr>
<td>hsa-miR-557</td>
<td></td>
<td>3.92 × 10^{-7}</td>
<td>-2.0</td>
</tr>
<tr>
<td>hsa-miR-708</td>
<td></td>
<td>1.45 × 10^{-6}</td>
<td>-2.5</td>
</tr>
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<td>hsa-miR-29c*</td>
<td></td>
<td>4.40 × 10^{-6}</td>
<td>-1.9</td>
</tr>
<tr>
<td>hsa-miR-617</td>
<td></td>
<td>1.49 × 10^{-5}</td>
<td>-1.5</td>
</tr>
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<td>hsa-miR-647</td>
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<td>-1.4</td>
</tr>
<tr>
<td>hsa-let-7c</td>
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<td>4.95 × 10^{-5}</td>
<td>-1.2</td>
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<td>hsa-miR-202</td>
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<td>6.55 × 10^{-5}</td>
<td>-2.0</td>
</tr>
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</table>

NOTE: Fold change indicates expression of adrenocortical carcinomas relative to adrenocortical adenomas. A positive fold change denotes upregulation, whereas a negative fold change indicates downregulation of the microRNA in adrenocortical carcinomas compared with adrenocortical adenomas. Abbreviations: ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; miRNA, microRNA.

Differences between groups were assessed statistically using REST-XL version 2 (Relative Expression Software Tool; ref. 20), wherein relative expression ratios are computed based on the PCR efficiency and crossing point differences.

**Statistical analysis.** The microarray data was analyzed using t test and Bonferroni correction such that differential expression was considered to be significant with a P < 0.0001. All other statistical analyses were done with SPSS 16 for Windows, and P < 0.05 was considered significant. Categorical data were analyzed using Fisher's exact test. The Mann-Whitney test was used for quantitative reverse transcriptase-PCR statistical analysis because the data were not normally distributed and were heteroskedastic, despite attempted data transformation. Univariate analysis of Kaplan-Meier curves was done using the log-rank test. Microarray data for miR-195 and miR-483-5p were dichotomized, with high and low expression defined with reference to the median expression of the microRNA levels in the tumor samples.

### Results

**Patients for microarray analysis.** MicroRNA microarray profiling was done on a cohort of 27 adrenocortical adenomas, 22 adrenocortical carcinomas, and six normal adrenal cortices. The clinical characteristics of adrenocortical adenoma and adrenocortical carcinoma subjects are shown in Table 1. All RNA samples with reasonable quality were included. One sample with RNA integrity number (21) of <5.0, as determined on the Agilent Bioanalyzer 2100 (Agilent), was excluded. Fifty-five samples with RNA integrity number of >7.0 and one with a RNA integrity number of 5.9 were included. Included in the analysis were 19 primary tumors. A single metastasis (cervical lymph node) from one subject whose primary tumor was also included and two other metastatic lesions (liver and adrenal bed recurrence) for which the primary tumors were not available were included in the analysis. Of the 21 adrenocortical tumors diagnosed histologically as adrenocortical carcinomas using the Weiss score, 10 had subsequently died of their disease, one had died from an unrelated cause, one is alive with disease...
Fig. 1.  

A, unsupervised hierarchical clustering of microRNAs and samples. Each row, a microRNA; each column, a sample. The microRNA clustering tree is on the left, and the sample clustering tree is at the top. The color scale at the bottom illustrates the relative expression level of a microRNA across all samples. Red, expression level above mean; blue, expression lower than the mean. The clustering is done on log $2(\text{Hy3}/\text{Hy5})$ ratios, which passed the filtering criteria across all samples; SD, $<1.0$. The heat map contains 66 microRNAs present in $>50$ of 56 samples. A, adrenocortical adenoma; C, adrenocortical carcinoma. The patient with the outlier adrenocortical carcinoma (at sign) has remained disease free 7 y after her operation. The single subject for whom the primary and metastatic cervical lymph node samples were available showed concordant pattern of microRNA expression (asterisk, primary tumor; number sign, metastatic lesion). B, chromosomal locations of significantly differentially expressed microRNAs between adrenocortical carcinomas and adrenocortical adenomas. Each green bar is an overexpressed microRNA, whereas each red bar signifies an underexpressed microRNA.
We selected four microRNAs for further validation. Table 3. List of significantly differentially expressed microRNAs between adrenocortical tumors and normal adrenal cortices

<table>
<thead>
<tr>
<th>microRNA</th>
<th>P</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-7</td>
<td>2.97 x 10^{-12}</td>
<td>-10.1</td>
</tr>
<tr>
<td>hsa-miR-129-3p</td>
<td>4.25 x 10^{-11}</td>
<td>-5.7</td>
</tr>
</tbody>
</table>

NOTE: Fold change indicates expression of adrenocortical tumors relative to normal adrenal cortices. Both significantly differentially expressed microRNAs were downregulated in adrenocortical tumors compared with normal, as indicated by the negative fold change.

Discussion

The present study is the first to describe a distinct microRNA expression signature in adrenocortical tumors that differentiates adrenocortical carcinomas from adrenocortical adenomas and normal adrenal tissue. A recent article described 33 significantly upregulated and 11 significantly downregulated microRNAs in primary pigmented nodular adrenocortical disease compared with normal adrenal tissue (27). More than 50 microRNAs are differentially expressed between adrenocortical carcinomas and adrenocortical adenomas, with 23 retaining significance after correction for multiple comparisons. A number of microRNAs, including miR-335, miR-106b, and miR-143, that are significantly
cortical adenomas on microarray analysis. miR-7 was the most significantly differentially expressed microRNA between adrenocortical tumors and normal adrenal cortex but was not differentially expressed between adrenocortical carcinomas and adrenocortical adenomas. miR-195, miR-335, and miR-7 have also been reported in the literature to be involved in the pathogenesis of other cancers (24-26). miR-483 was selected for validation because it is located within intron 2 of IGF2, a gene that has been shown by a number of studies to be upregulated in adrenocortical carcinomas compared with adrenocortical adenomas.

To confirm results obtained by microarray analysis, quantitative reverse transcriptase-PCR was used to quantify levels of miR-195, miR-335, miR-483, and miR-7 in 10 adrenocortical carcinomas and nine adrenocortical adenomas. Log mean expression of each microRNA for adrenocortical carcinomas and adrenocortical adenomas is shown in Fig. 2A, and miR-7 expression in adrenocortical carcinomas, adrenocortical adenomas, and normal samples is shown in Fig. 2B. For miR-195 and miR-335, differential expression was confirmed in these quantitative reverse transcriptase-PCR analyses such that both were significantly downregulated in adrenocortical carcinomas compared with adrenocortical adenomas (Fig. 2A). There was a trend toward miR-483 being significantly upregulated in adrenocortical carcinomas compared with adrenocortical adenomas, but this did not reach statistical significance (Fig. 2A). Expression of miR-7 differed significantly not only between adrenocortical tumors (either adrenocortical adenoma or adrenocortical carcinoma) and normal but also in this analysis between adrenocortical carcinomas and adrenocortical adenomas (Fig. 2B). Results for the external independent validation cohort for quantitative reverse transcriptase-PCR of six adrenocortical carcinomas and five adrenocortical adenomas for miR-195, miR-335, and miR-483, and three adrenocortical carcinomas and three adrenocortical adenomas for miR-7 are shown in Fig. 2C. Only miR-195 and miR-7 were significantly different in this independent cohort. This could be a result of the small numbers of this group.

Kaplan-Meier analysis of miR-195 and miR-483. Survival data were available for 18 of the 21 patients classified as adrenocortical carcinoma by Weiss score. Kaplan-Meier analysis was done using microarray data for miR-195, miR-335, miR-483-5p, and miR-7. The specific overall survival for dichotomized data of miR-195 (Fig. 3A) and miR-483-5p (Fig. 3B) were significantly different. Log-rank comparison of specific overall survival in dichotomized expression of miR-195 and miR-483-5p individually and in combination (Fig. 3C) confirmed significantly different outcome in the two groups.

Discussion

The present study is the first to describe a distinct microRNA expression signature in adrenocortical tumors that differentiates adrenocortical carcinomas from adrenocortical adenomas and normal adrenal tissue. A recent article described 33 significantly upregulated and 11 significantly downregulated microRNAs in primary pigmented nodular adrenocortical disease compared with normal adrenal tissue (27). More than 50 microRNAs are differentially expressed between adrenocortical carcinomas and adrenocortical adenomas, with 23 retaining significance after correction for multiple comparisons. A number of microRNAs, including miR-335, miR-106b, and miR-143, that are significantly

reurrence, seven have remained disease free, and two have been lost to follow-up.

Microarray analysis. We carried out two separate preplanned analyses using the microarray data. In the first, microRNA expression was compared between adrenocortical carcinomas and adrenocortical adenomas. We found that 23 microRNAs were significantly differentially expressed with $P<0.0001$ after correcting for multiple comparisons. Fourteen were significantly upregulated, whereas nine were significantly downregulated in adrenocortical carcinomas compared with adrenocortical adenomas (Table 2). Five of the six normals form a cluster between adrenocortical carcinomas and adrenocortical adenomas. The patient with the outlier adrenocortical carcinoma (at sign) has remained disease free 7 years after her operation. Notably, this sample was also grouped with the adrenocortical adenomas instead of the adrenocortical carcinomas on mRNA gene expression profiling (6). It is intriguing to suggest that this tumor may be considered a false positive of the Weiss system and conventional histopathologic analysis rather than a false negative of mRNA and microRNA expression. Interestingly, the adrenocortical carcinomas tend to form two distinct groups. There are, however, patients in both groups that have died of their disease.

The single subject for whom the primary and metastatic cervical lymph node samples were available showed concordant pattern of microRNA expression (in Fig. 1A, the primary tumor is denoted by an asterisk and the metastatic lesion by a number sign).

The chromosomal locations of the significantly differentially expressed microRNAs between adrenocortical carcinomas and adrenocortical adenomas are shown in Fig. 1B. These chromosomal regions do not overlap with previously identified areas of large-scale losses or gains in adrenocortical carcinomas analyzed by comparative genomic hybridization (22, 23).

Our second preplanned analysis of microRNA array data was to compare adrenocortical tumors (i.e., adrenocortical carcinomas and adrenocortical adenomas combined) with normal samples. The number of significantly differentially expressed microRNAs was smaller when comparison was made with normal adrenal cortex. After correcting for multiple comparisons, two microRNAs were significantly differentially expressed between adrenocortical tumors versus normal adrenal cortices (Table 3).

Quantitative reverse transcriptase-PCR validation of microRNAs. We selected four microRNAs for further validation by quantitative reverse transcriptase-PCR. miR-195, miR-335, and miR-483 were three of the top six most significantly differentially expressed between adrenocortical carcinomas and adreno-
deregulated in adrenocortical carcinomas compared with adrenocortical adenomas have previously been reported to be deregulated in other cancers. For example, miR-195 is downregulated in squamous cell carcinomas of the tongue compared with normal tongue (25), and miR-335 has been reported to be significantly underexpressed in breast cancer compared with normal breast tissue (24).

miR-195 is significantly downregulated in adrenocortical carcinomas compared with adrenocortical adenomas. miR-195 has also been shown to have a 3-fold downregulation in squamous cell carcinomas of the tongue compared with normal tongue (25). It is located on 17p13.1, within a 10.4-megabase common minimal region of loss, which we have previously described in adrenocortical carcinomas but not adrenocortical adenomas (18). miR-195 belongs to the miR-15/miR-16 family. First described in chronic lymphocytic leukemia, miR-15 and miR-16 were identified as the only genes within the smallest common region of the 13q14 deletion and subsequently found to be downregulated in >50% of chronic lymphocytic leukemia (28). In chronic lymphocytic leukemia cases, an inverse correlation was found between miR-15 and miR-16 and B cell lymphoma 2 (BCL2). In a leukemic cell line model, it was

![Fig. 2. Quantitative reverse transcriptase-PCR validation of microarray results. A, quantitative reverse transcriptase-PCR result for miR-195, miR-335, and miR-483 in adrenocortical carcinomas and adrenocortical adenomas. The data are expressed as logarithmic mean expression relative to a pooled sample of four normal adrenal cortices. Data are mean ± SE (adrenocortical carcinoma, n = 10; adrenocortical adenoma, n = 9). Statistical significance was determined with the Mann-Whitney test. Each sample was run in triplicate and repeated in two separate experiments. B, quantitative reverse transcriptase-PCR result for miR-7 comparing adrenocortical carcinomas with normal adrenal cortex, adrenocortical adenomas with normal adrenal cortex, and adrenocortical carcinomas with adrenocortical adenomas. The data are expressed as logarithmic mean expression relative to a pooled sample of one adrenocortical carcinoma, one adrenocortical adenoma, and one normal adrenal cortex. Data are mean ± SE (adrenocortical carcinoma, n = 7; adrenocortical adenoma, n = 6; normal adrenal cortices, n = 6). Statistical significance was determined using the Mann-Whitney test. Expression of miR-7 by quantitative reverse transcriptase-PCR is significantly different between adrenocortical carcinoma and normal (P = 0.032), adrenocortical carcinoma and adrenocortical adenoma (P = 0.002), and adrenocortical adenoma and normal (P = 0.002). Each sample was run in triplicate and repeated in two separate experiments. C, quantitative reverse transcriptase-PCR results for external validation cohort. For miR-195, miR-335, and miR-483, there are six adrenocortical carcinomas and five adrenocortical adenomas, whereas for miR-7, there are three adrenocortical carcinomas and three adrenocortical adenomas in the external validation cohort. Each sample was run in triplicate and repeated in two separate experiments. For this cohort, miR-195 and miR-7 are significantly differentially expressed between adrenocortical carcinomas and adrenocortical adenomas.](#)
found that overexpression of miR-15 and miR-16 resulted in repression of BCL2 and induction of apoptosis (29).

Our microarray data show that miR-335 is significantly downregulated in adrenocortical carcinomas compared with adrenocortical adenomas. This result was confirmed by quantitative reverse transcriptase-PCR. Located on 7q32.2, miR-335 is reported to be a tumor suppressor gene in breast cancer (30). Tavazoie et al. (24) showed that miR-335 inhibits metastasis and migration by targeting SOX4 and the extracellular matrix component tenascin C.

By microarray analysis with validation by quantitative reverse transcriptase-PCR, miR-483 is upregulated in adrenocortical carcinomas compared with adrenocortical adenomas, but the results did not reach statistical significance. Located on 11p15.5, miR-483 is located in intron 2 of IGF2. Because IGF2 is frequently upregulated in adrenocortical carcinomas...
compared with adenocortical adenomas, the overexpression of miR-483 could be a bystander effect from the upregulation of IGF2 in adenocortical carcinomas. An alternate hypothesis for this phenomenon, which occurs in nearly half of overexpressed microRNA, is that the intronic microRNA silences genes whose function are antagonistic to the function of their host gene (31).

We found that miR-7 is significantly downregulated in adenocortical carcinomas compared with normal adrenal cortex and adenocortical adenomas compared with normal adrenal cortex. These results were confirmed by quantitative reverse transcriptase-PCR. We also found that expression of miR-7 is significantly lower in adenocortical carcinomas compared with adenocortical adenomas on quantitative reverse transcriptase-PCR, a result that was not found on microarray analysis. miR-7 is a potential tumor suppressor in the pathogenesis of glioblastoma. It has been found to be downregulated in glioblastomas compared with surrounding normal brain tissue. miR-7 suppresses epidermal growth factor receptor expression and inhibited the Akt pathway. Transfection of glioblastoma cell lines with miR-7 decreased their viability and invasiveness (26).

Other microRNAs known to be involved with tumorigenesis were also found to be differentially expressed in adenocortical carcinomas compared with adenocortical adenomas. These include miR-106b and miR-143. The microRNAs in the miR-106b family have been found to be overexpressed in a number of different tumors. They are thought to contribute to tumor cell proliferation in part by regulating cell cycle progression (32). Bloomston et al. (33) found that miR-143 is upregulated 2.19 times in pancreatic cancer compared with normal pancreas. Interestingly, miR-143 is significantly downregulated in colorectal cancer compared with normal adjacent tissue (12, 34).

There have been two recent microarray gene expression profiling studies published that have concentrated on dividing adenocortical carcinomas into two groups based on prognosis. Giordano et al. (5) found that 85 differentially expressed genes involved in proliferation and cell cycle are able to divide adenocortical carcinomas into predominantly high- and low-grade groups with poorer survival in the former group. Studying 92 adenocortical tumors by microarray analysis, de Reynies et al. (4) found that budding uninhibited by benzimidazoles 1 homolog β (BUB1B) and PTEN-induced putative kinase 1 (PINK1) can divide adenocortical carcinomas into good and poor outcome groups. We did Kaplan-Meier analysis using the four validated microRNAs from the original profiling cohort. We found that adenocortical carcinomas with lower expression of miR-195 or higher expression of miR-483 had shorter overall specific survival, whereas the expression of miR-335 or miR-7 was not associated with adenocortical carcinoma outcome. Combining low expression of miR-195 and high expression of miR-483-5p yielded the same results as either of these microRNAs individually. At present, there are no good predictors of outcome for adenocortical carcinomas. Our results suggest that expression levels of miR-195 and miR-483-5p can be used as predictors of poorer survival in patients with adenocortical carcinoma and identify those patients requiring more aggressive initial multimodal therapy that are currently the subject of randomized trials (35).

Conclusions. In this study, we found a number of microRNAs that are significantly differentially expressed between adenocortical carcinomas, adenocortical adenomas, and normal adrenal cortex, implying that microRNAs are involved in the pathogenesis of adenocortical tumors. miR-195 and miR-335 are significantly downregulated in adenocortical carcinomas compared with adenocortical adenomas. The one case wherein microRNA and conventional histologic analysis were discordant behaved in a benign manner predicted by the microRNA profile, suggesting that microRNA analysis may be equally sensitive and more specific than the Weiss score in predicting biological behavior of adenocortical tumors. This may have diagnostic significance in adenocortical tumors with borderline histologic features. Low expression of miR-195 and high expression of miR-483-5p predict for poor overall specific survival in adenocortical carcinomas and may have a role in identifying a cohort of adenocortical carcinoma patients who require aggressive multimodal therapy at diagnosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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miR-195 and miR-483-5p Identified as Predictors of Poor Prognosis in Adrenocortical Cancer

Patsy Siok Hwa Soon, Lyndal J. Tacon, Anthony J. Gill, et al.


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