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Molecular Classification of Renal Tumors by Gene Expression Profiling

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Renal tumor classification is important because histopathological subtypes are associated with distinct clinical behavior. However, diagnosis is difficult because tumor subtypes have overlapping microscopic characteristics. Therefore, ancillary methods are needed to optimize classification. We used oligonucleotide microarrays to analyze 31 adult renal tumors, including clear cell renal cell carcinoma (RCC), papillary RCC, chromophobe RCC, oncocytoma, and angiomyolipoma. Expression profiles correlated with histopathology; unsupervised algorithms clustered 30 of 31 tumors according to appropriate diagnostic subtypes while supervised analyses identified significant, subtype-specific expression markers. Clear cell RCC overexpressed proximal nephron, angiogenic, and immune response genes, chromophobe RCC oncocytoma overexpressed distal nephron and oxidative phosphorylation genes, papillary RCC overexpressed serine protease inhibitors, and extracellular matrix products, and angiomyolipoma overexpressed muscle developmental, lipid biosynthetic, melanocytic, and distinct angiogenic factors. Quantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry of formalin-fixed renal tumors confirmed overexpression of proximal nephron markers (megalin/low-density lipoprotein-related protein 2, α-methylacyl CoA racemase) in clear cell and papillary RCC and distal nephron markers (β-defensin 1, claudin 7) in chromophobe RCC/oncocytoma. In summary, renal tumor subtypes were classified by distinct gene expression profiles, illustrating tumor pathobiology and translating into novel molecular bioassays using fixed tissue. *(J Mol Diagn 2005, 7:206–218)*

Renal cell carcinoma (RCC) is the most common malignancy of the adult kidney, comprising 3% of all human cancers.[1] Localized tumors can be detected by abdominal imaging and cured by surgery.[2] However, 25 to 40% of cases present with extrarenal growth or metastases,[3] and one-third of apparently localized lesions develop metastases during the postoperative course.[4,5] Advanced RCC responds poorly to systemic therapy and has a 5-year survival rate of less than 10%.[6,7]

Important predictors of outcome for RCC include tumor stage, Fuhrman nuclear grade, histopathological classification, and perioperative thrombocytosis.[3,8–10] The current renal tumor classification system is based on morphology, as well as underlying genetic differences.[3,11,12] More than 90% of clinically significant lesions can be diagnosed as one of the common subtypes of renal epithelial tumor: clear cell (conventional) RCC, papillary RCC, chromophobe RCC, and renal oncocytoma. Mesenchymal renal tumors are more rare and include angiomyolipoma. Recent clinicopathological surveys have indicated that clear cell RCC has the highest rate of metastasis and poorest survival among common renal malignancies.[3] Papillary and chromophobe carcinomas are relatively indolent but exhibit potential for metastasis and transformation to high-grade, sarcomatoid tumors. In contrast, typical oncocytomas and angiomyolipomas are considered benign neoplasms.

Renal tumor subtypes exhibit several common morphological characteristics, making diagnosis difficult and subjective in many cases. For instance, eosinophilic variants of clear cell and chromophobe RCC can closely resemble the benign oncocytoma histologically. Therefore, ancillary molecular methods are needed for optimal diagnosis and clinical management. In several types of human cancer, gene expression microarrays have proved to be effective tools for classifying tumors and identifying novel molecular biomarkers.[13–16] Previously, we used microarrays to profile gene expression in clear cell RCC, papillary RCC, chromophobe RCC, and onco-

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cytoma; based on distinct expression patterns, we developed a novel immunohistochemical panel for renal tumor subtyping.[17,18] Other groups have confirmed these findings in larger studies.[5,19–21] We extended our observations in the current experiments, using oligonucleotide microarrays to profile the expression of several thousand genes in a cohort of clear cell RCC, papillary RCC, chromophobe RCC, oncocytoma, and angiomyolipoma. We validated the microarray data for selected, differentially expressed gene products in an independent cohort of fixed tissues, using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry, and thereby confirmed these gene products as potential expression markers for renal tumor classification.

Materials and Methods

Experimental Specimens

Microarray experiments were performed on frozen specimens from 13 clear cell RCC, 5 papillary RCC, 4 chromophobe RCC, 3 oncocytoma, and 6 angiomyolipoma. Clinicopathological characteristics of this tumor cohort are described in Supplementary Table 1 at *http://jmd.amjpathol.org*. Quantitative RT-PCR experiments were performed on formalin-fixed paraffin-embedded tissue from an independent cohort of 10 clear cell RCC, 6 papillary RCC, 5 chromophobe RCC, and 7 renal oncocytoma. Immunohistochemistry was performed on a fixed tissue microarray that included 33 clear cell RCC, 19 papillary RCC, 6 chromophobe RCC, and 6 oncocytoma. The Emory University and Atlanta Veterans Affairs Medical Center Departments of Pathology and Laboratory Medicine diagnosed tumors using published criteria:[3] clear cell RCC– neoplastic clear cells with an anastomosing vascular network; papillary RCC– circumscription with a fibrous capsule, papillary growth pattern, foam cells, and necrosis; chromophobe RCC– broad alveolar or nested growth pattern, neoplastic cells with irregular nuclei and perinuclear halos, clear flocculent or granular eosinophilic cytoplasm; renal oncocytoma– circumscription with a central scar, nested or tubulocystic growth pattern, and neoplastic oncocytes with small round nuclei and granular eosinophilic cytoplasm; angiomyolipoma– mesenchymal tumor containing mature adipose, spindle, or epithelioid smooth muscle cells and thick-walled blood vessels. Carcinoma grading and staging were based on the standard Fuhrman nuclear grading system and the Tumor-Node-Metastasis staging system (TNM, International Union Against Cancer), respectively.[3,8,11] The Emory University Institutional Review Board approved this research under protocol 255-2002.

Microarray Hybridization

Frozen tumor specimens were homogenized in 10 vol of TRIzol (Invitrogen, Carlsbad, CA) per g of tissue. Total RNA was isolated using the standard TRIzol protocol and purified further with the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. RNA was quantified and assessed for integrity using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Expression profiles of all specimens were compared to a commercial universal reference RNA (Clontech, Palo Alto, CA). Probe synthesis and microarray hybridization were performed according to standard Affymetrix protocols, described in detail at the Internet site *http://www.affymetrix.com/community/academic/grant.affx*. Briefly, total RNA (5 μg per specimen) was reverse-transcribed into double-stranded cDNA, and biotin-labeled cRNA was produced by *in vitro* transcription. Labeled cRNA was fragmented and digested by DNase I before hybridization. Hybridization cocktails were prepared by combining fragmented targets, probe array controls, bovine serum albumin, and herring sperm DNA. Cocktails were applied to HG Focus oligonucleotide microarrays (Affymetrix, Santa Clara, CA) for 16 hours, followed by automated washing and staining on an Affymetrix workstation. After staining, microarrays were scanned and analyzed with Affymetrix Microarray Suite 5.0 software, to define probe cells, compute signal intensities in each cell, and calculate signal $\log_2$ expression ratios for each gene in tumor versus reference specimens. The HG Focus arrays produced data for 8746 genes. All hybridization experiments met the following quality control criteria: average background, $<$100 U; noise, $<$5 U; 3′:5′ ratio of control genes, $<$3; and RNA spikes present with appropriate signal intensities. Scaling factors and transcript presence rates varied less than 20% among tumor samples.

Data Analysis

Affymetrix data sets were normalized with a robust multiarray algorithm[22] accounting for GC sequence information (GCRMA algorithm), encoded in GeneTraffic software (Iobion Informatics, La Jolla, CA). Expression profiles were filtered to exclude genes with fewer than two observations of absolute value $\log_2$ ratio $>$2, or with mean $\log_2$ ratio range (maximum $-$ minimum) $<$ 2. This procedure selected 4030 differentially expressed genes from the total of 8746. To compare global expression patterns among renal tumor subtypes, the filtered expression profiles were analyzed by unsupervised hierarchical average linkage clustering, using Pearson correlation as the similarity metric.[23] To identify genes that correlated significantly with renal tumor subtypes, the unfiltered expression profiles were analyzed with a supervised significance analysis of microarrays (SAM) algorithm,[24] using the following parameters: data type = two-class unpaired (ie, one tumor subtype versus all other tumors); imputer engine = 10-nearest neighbor; fold change cutoff = 2.0; permutation number = 500; random number generator seed = 1234567; and median false discovery rate $<$1%, corresponding to Δ = 1.12, 1.79, 1.44, and 1.53 for clear cell RCC, chromophobe RCC/renal oncocytoma, papillary RCC, and angiomyolipoma, respectively. To determine whether biological processes were highly represented in renal tumor expression profiles, the significant

Table 1. Immune Response and Angiogenesis Genes Overexpressed in Clear Cell Renal Cell Carcinoma

Symbol	UniGene	GenBank	Name
Gene ontology: immune response (GO:0006955; $P = 1.0 \times 10^{-56}$)			
AOAH	Hs.82542	NM_001637	Acyloxyacyl hydrolase precursor
APOL3	Hs.241535	NM_014349	Apolipoprotein L3 isoform 2
ARHGDIB	Hs.83656	NM_001175	Rho GDP dissociation inhibitor (GDI) β
BST2	Hs.118110	NM_004335	Bone marrow stromal cell antigen 2
C1QA	Hs.9641	NM_015991	Complement component 1, q, α
C1QB	Hs.8986	NM_000491	Complement component 1, q, β
C1QR1	Hs.97199	NM_012072	Complement component 1, q, receptor 1
C1R	Hs.1279	AL573058	Complement component 1, r
C1S	Hs.434029	M18767	Complement component 1, s
C3	Hs.284394	NM_000064	Complement component 3 precursor
CCL19	Hs.50002	U88321	Small inducible cytokine A19 precursor
CCL2	Hs.303649	S69738	Small inducible cytokine A2 precursor
CCL20	Hs.75498	NM_004591	Chemokine (C-C motif) ligand 20
CCL4	Hs.75703	NM_002984	Chemokine (C-C motif) ligand 4 precursor
CCR1	Hs.301921	AI421071	Chemokine (C-C motif) receptor 1
CCR2	Hs.395	NM_000647	Chemokine (C-C motif) receptor 2 isoform A
CCR5	Hs.54443	NM_000579	Chemokine (C-C motif) receptor 5
CCR7	Hs.1652	NM_001838	Chemokine (C-C motif) receptor 7 precursor
CD163	Hs.74076	NM_004244	CD163 antigen
CD2	Hs.89476	NM_001767	CD2 antigen (p50), sheep red blood cell receptor
CD3D	Hs.95327	NM_000732	CD3D antigen, δ polypeptide (TiT3 complex)
CD53	Hs.82212	NM_000560	CD53 antigen
CD74	Hs.84298	K01144	Invariant γ chain
CD8A	Hs.85258	AW006735	CD8 antigen alpha polypeptide isoform 1 precursor
CSF1R	Hs.174142	NM_005211	Colony-stimulating factor 1 receptor precursor
CSF2RB	Hs.285401	AV756141	Colony-stimulating factor 2 receptor, β, low-affinity
CST7	Hs.143212	AF031824	Cystatin F
CXCL10	Hs.2248	NM_001565	Small inducible cytokine B10 precursor
CXCL12	Hs.237356	NM_000609	Chemokine (C-X-C motif) ligand 12
CXCL14	Hs.24395	NM_004887	Small inducible cytokine B14 precursor
CXCL9	Hs.77367	NM_002416	Small inducible cytokine B9 precursor
ENTPD1	Hs.205353	AV717590	Ectonucleoside triphosphate diphosphohydrolase 1
F8	Hs.79345	NM_000132	Coagulation factor VIII isoform a precursor
FCER1G	Hs.433300	NM_004106	Fc fragment of IgE, high affinity I, receptor for, γ
FCGR2A	Hs.78864	NM_021642	Fc fragment of IgG, low affinity IIa, receptor for (CD32)
FCGR3A	Hs.176663	J04162	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)
FOS	Hs.25647	BC004490	*c*-fos
FPR1	Hs.753	NM_002029	Formyl peptide receptor 1
G1P2	Hs.432233	NM_005101	Interferon, α-inducible protein (clone IFI-15K)
GZMA	Hs.90708	NM_006144	Granzyme A precursor
HF1	Hs.250651	X04697	H factor 1 (complement)
HLA-DMB	Hs.1162	NM_002118	Major histocompatibility complex, class II, DM β
HLA-DPB1	Hs.814	NM_002121	Major histocompatibility complex, class II, DP β 1
HLA-DQA1	Hs.198253	BG397856	Major histocompatibility complex, class II, DQ α 1
ICSBP1	Hs.14453	AI073984	Interferon consensus sequence-binding protein 1
IFITM1	Hs.458414	MGC27165	Interferon-induced transmembrane protein 1 (9–27)
IGJ	Hs.76325	AV733266	Immunoglobulin J polypeptide
IGSF6	Hs.135194	NM_005849	Immunoglobulin superfamily, member 6
IL10RB	Hs.173936	BC001903	Interleukin 10 receptor, β precursor
IL1R2	Hs.25333	NM_004633	Interleukin 1 receptor, type II precursor
IL2RB	Hs.75596	NM_000878	Interleukin 2 receptor β precursor
IL2RG	Hs.84	NM_000206	Interleukin 2 receptor, γ chain, precursor
IL6	Hs.93913	NM_000600	Interleukin 6 (interferon, β 2)
IL7R	Hs.362807	NM_002185	Interleukin 7 receptor precursor
INHBB	Hs.1735	NM_002193	Inhibin β B subunit precursor
IRF1	Hs.80645	NM_002198	Interferon regulatory factor 1
IRF7	Hs.166120	NM_004030	Interferon regulatory factor 7 isoform a
ITGB2	Hs.83968	NM_000211	Integrin β chain, β 2 precursor
ITK	Hs.211576	D13720	IL2-inducible T-cell kinase
JAG2	Hs.166154	AF029778	Jagged 2 isoform a precursor
LCP2	Hs.2488	AI123251	Lymphocyte cytosolic protein 2
LIF	Hs.2250	NM_002309	Leukemia inhibitory factor
LTB	Hs.890	NM_002341	Lymphotoxin-β isoform a
MNDA	Hs.153837	NM_002432	Myeloid cell nuclear differentiation antigen
MX1	Hs.76391	NM_002462	Myxovirus resistance protein 1

(*Table continues*)

Table 1. *Continued*

Symbol	UniGene	GenBank	Name
MX2	Hs.926	NM_002463	Myxovirus resistance protein 2
NK4	Hs.943	NM_004221	Natural killer cell transcript 4
PSMB10	Hs.9661	NM_002801	Proteasome β 10 subunit proprotein
PSMB9	Hs.381081	NM_002800	Proteasome β 9 subunit isoform 1 proprotein
TCIRG1	Hs.46465	NM_006019	T cell, immune regulator 1, isoform a
TLR2	Hs.63668	NM_003264	Toll-like receptor 2
TLR3	Hs.29499	NM_003265	Toll-like receptor 3
TLR7	Hs.179152	NM_016562	Toll-like receptor 7
TNFSF7	Hs.99899	NM_001252	Tumor necrosis factor ligand superfamily, member 7
TYROBP	Hs.9963	NM_003332	TYRO protein tyrosine kinase binding protein
UBD	Hs.44532	NM_006398	Diubiquitin
Gene ontology: angiogenesis (GO:0001525; $P = 8.5 \times 10^{-3}$)			
ANGPT2	Hs.115181	AF187858	Angiopoietin 2
ANGPTL4	Hs.9613	NM_016109	Angiopoietin-like 4 protein
FLT1	Hs.381093	AA058828	Vascular endothelial growth factor receptor 1
KDR	Hs.12337	NM_002253	Vascular endothelial growth factor receptor 2
VEGF	Hs.73793	AF022375	Vascular endothelial growth factor
VEGFC	Hs.79141	U58111	Vascular endothelial growth factor C

The significance analysis of microarrays (SAM) identified genes overexpressed in clear cell RCC versus all other tumors. The GOstat program identified gene ontology functional annotation terms that were statistically overrepresented in the clear cell RCC expression profile.

expression markers identified by SAM were analyzed with the GOstat program (*http://gostat.wehi.edu.au/*).[25] This program compiled functional annotations from the Gene Ontology Consortium database[26] that were associated with gene lists identified by SAM. Frequencies of specific gene ontology associations were compared in the differentially expressed gene lists versus the entire list of genes featured on HG Focus microarrays. The Fisher's exact test identified gene ontology terms overrepresented among renal tumor differential expression profiles, using a Benjamini false discovery rate correction for multiple testing.

Quantitative RT-PCR

Experiments were performed according to published protocols with minor modifications.[27] Briefly, histological sections were deparaffinized with ethanol and xylene, and cells of interest were microdissected with a sterile scalpel. Tissue was digested in buffer containing proteinase K at 55°C overnight, and total RNA was isolated by phenol/chloroform extraction. The sample was treated with DNase to minimize contamination with genomic DNA. Fluorogenic quantitative RT-PCR assays were performed in triplicate with standard SYBR Green methodology on the I-cycler system (Bio-Rad, Hercules, CA). Reaction specificity was assessed by melting point analyses, in which single melting point peaks were required at temperatures predicted by amplicon sequence. Reactions without reverse transcription and template served as controls for DNA contamination and specimen carryover. The following test genes were analyzed: megalin/low-density lipoprotein-related protein 2 (*LRP2*: forward primer, 5′-gctgataaaacgagacgcacagta; reverse primer, 5′-aggacggaaccaatcagtgaag); β-defensin 1 (*DEFB1*: forward primer, 5′-tttactctctgcttacttttgtctgagatg; reverse primer, 5′-tgctgacgcaattgtaatgatca); and α-methylacyl CoA racemase (*AMACR*: forward primer, 5′-gggtcaggtcattgatgcaaa; reverse primer, 5′-ttcccacagactcaatttctgagtt). All primer pairs were intron-spanning, and were developed by analysis of intron/exon structures in the Ensembl sequence database (*www.ensembl.org*), followed by entry of appropriate sequences in Primer Express software (Applied Biosystems, Foster City, CA). Test gene expression was normalized to 28S ribosomal RNA and referenced to a standard RNA specimen. Relative normalized gene expression was compared in renal tumor subtypes, with statistical significance assessed by analysis of variance.

Immunohistochemistry

Tissue microarrays were constructed from fixed renal tumor core biopsies, 0.6 mm in diameter and 3 to 4 mm in height, which were placed in recipient paraffin blocks (45.0 × 20.0 mm) with a tissue arrayer (Beecher Instruments, Silver Spring, MD). Three to four tissue cores were arrayed per case. Tissue microarray sections (5 μm) were dewaxed, and steam antigen retrieval was performed at pH 6.5 in a pressure cooker for 20 minutes.[28] Tissue sections were incubated with mouse monoclonal antibody against the claudin-7 gene product (*CLDN7*) for 25 minutes at room temperature (1:400 dilution; Zymed Corporation, South San Francisco, CA). After washing unbound antibody, sections were treated with goat anti-mouse immunoglobulin conjugated to a peroxidase-labeled polymer, according to the manufacturer's instructions (Envision kit; DAKO Corp., Carpinteria, CA). Immunohistochemical reactions were developed with diaminobenzidine as the chromogenic peroxidase substrate. Sections were counterstained with hematoxylin after immunohistochemistry. Specificity was verified by negative control reactions without primary antibody, as well as appropriate membranous staining reactions in positive control colon tissues. A positive reaction was defined as membranous staining on ≥10% tumor cells in one or more tissue cores. Frequency of reactive cases

was compared among renal tumor subtypes, using χ^2 analysis to assess for statistical significance.

Results

Expression patterns of 8746 genes were measured in 13 clear cell RCCs, 5 papillary RCCs, 4 chromophobe RCCs, 3 renal oncocytomas, and 6 angiomyolipomas using Affymetrix oligonucleotide microarrays. To characterize the unique expression profiles of renal tumor subtypes, unsupervised hierarchical average linkage clustering was used to group tumors and genes by similarity in expression profiles. The resulting molecular classification correlated strongly with histopathology; 30 of 31 tumors were clustered according to appropriate diagnostic subtypes (Figure 1, *x* axis dendrogram). The sole outlier was a high-grade papillary carcinoma with sarcomatoid transformation, which was clustered with clear cell RCC. The SAM procedure identified genes with the strongest correlation to specific tumor subtypes, and the GOstat program determined the statistical representation of specific gene ontology functional annotation terms in the gene lists identified by SAM. Clear cell RCC overexpressed 402 and underexpressed 220 genes, at a median false discovery rate of 0.99%. As shown in Table 1, clear cell tumors overexpressed a significant number of immune response genes ($P = 1.0 \times 10^{-36}$) and angiogenic factors ($P = 8.5 \times 10^{-3}$). Chromophobe RCC and oncocytoma overexpressed 510 and underexpressed 479 genes, at a median false discovery rate of 0.92%. As shown in Table 2, the overexpressed sequences included a significant number related to electron transport ($P = 4.0 \times 10^{-14}$), oxidative phosphorylation ($P = 5.9 \times 10^{-9}$), and energy pathways ($P = 5.9 \times 10^{-9}$), whereas the underexpressed sequences included I kappa B kinase/nuclear factor-κB signaling activators ($P = 1.3 \times 10^{-3}$). Papillary RCC significantly overexpressed 95 genes and underexpressed only 1 gene, at a median false discovery rate of 0.80%. Although no gene ontology terms were statistically overrepresented in the papillary RCC expression profile, the list of significantly overexpressed genes included several encoding serine-type endopeptidase inhibitors and extracellular matrix products (Table 3). Angiomyolipoma overexpressed 409 and underexpressed 108 genes, at a median false discovery rate of 0.76%. As shown in Table 4, genes related to muscle development ($P = 3.6 \times 10^{-4}$), lipid biosynthesis ($P = 4.5 \times 10^{-3}$), and pigmentation ($P > 0.05$) were overexpressed in angiomyolipoma. In addition, these tumors expressed vascular endothelial growth factors B and D (*VEGFB*, *VEGFD*) at high levels. The complete microarray data are submitted on the National Center for Biotechnology Information Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo/*). Detailed results of the SAM and GOstat analyses are presented in Supplementary Tables 2 and 3 at *http://jmd.amjpathol.org.*

By microarray, clear cell and papillary RCC overexpressed markers of proximal nephron epithelium, such as cubilin (*CBLN*) and megalin/low-density lipoprotein-related protein 2 (*LRP2*). Papillary RCC specifically overexpressed the proximal nephron marker *AMACR*. In contrast, chromophobe RCC and oncocytoma overexpressed markers of distal nephron epithelium, such as parvalbumin (*PVALB*), chloride channels Ka and Kb (*CLCNKA*, *CLCNKB*), and *DEFB1* (see complete microarray data). To validate these findings, quantitative RT-PCR was performed in an independent cohort of fixed renal tumors to measure mRNA expression of *LRP2*, *AMACR*, and *DEFB1* (Figure 2). Consistent with the microarray data, *DEFB1* was overexpressed in chromophobe RCCs and renal oncocytomas versus clear cell and papillary RCCs ($P = 0.024$), and *AMACR* was overexpressed in papillary RCC versus all other tumors ($P = 0.0047$). *LRP2* was expressed at higher levels in clear cell and papillary RCCs versus chromophobe RCC and oncocytoma, although the difference did not reach statistical significance ($P = 0.30$).

Unsupervised clustering of the microarray data did not resolve chromophobe RCC from oncocytoma, indicating similar overall expression patterns. In addition, the SAM algorithm did not identify any individual genes that were expressed differentially between the two subtypes. Nevertheless, within limits set by the microarray study size, a small number of gene products showed a trend toward differential expression between chromophobe RCC and oncocytoma. For example, claudins 7 and 8 (*CLDN7*, *CLDN8*) were relatively overexpressed in chromophobe RCC (Figure 3a). To test the validity of this finding, immunohistochemistry for *CLDN7* was performed on a renal epithelial tumor tissue microarray (Figure 3b). Strong membranous staining was produced in the tumor cells of 4 of 6 chromophobe RCCs versus 2 of 6 oncocytomas. In papillary RCC, membranous staining of tumor cells was obtained in 4 of 19 cases, although the predominant staining pattern was cytoplasmic in stromal cells. In contrast, 0 of 33 clear cell RCC cases were positive for *CLDN7* gene product in tumor or stromal cells ($P \leq 0.001$, chromophobe RCC versus all other tumors).

Discussion

Until quite recently, the classification of adult renal tumors was limited to two major subtypes: clear cell and granular cell. Subsequent morphological and cytogenetic studies led to the recognition of several distinct renal tumor subtypes, culminating in the contemporary World Health Organization classification published in 2004.[29] The clinical relevance of this classification system is underscored by distinct prognoses associated with different renal tumor subtypes,[3,8,30] a finding that has prompted several proposals to account for histological subtype in the design of new therapies and clinical trials.[31–33] However, new approaches are needed for diagnosis and clinical management of renal tumors. Light microscopy, the standard method for diagnostic classification, is difficult because renal tumor subtypes share common histopathological features.[3,8,11] This problem is likely to grow with the advent of diagnostic procedures that result in small, distorted renal tumor biopsies.[34] Gene expression profiling is a promising approach to address this problem, because expression microarrays can resolve certain tumors into diagnostic, prognostic, and therapeutic subclasses

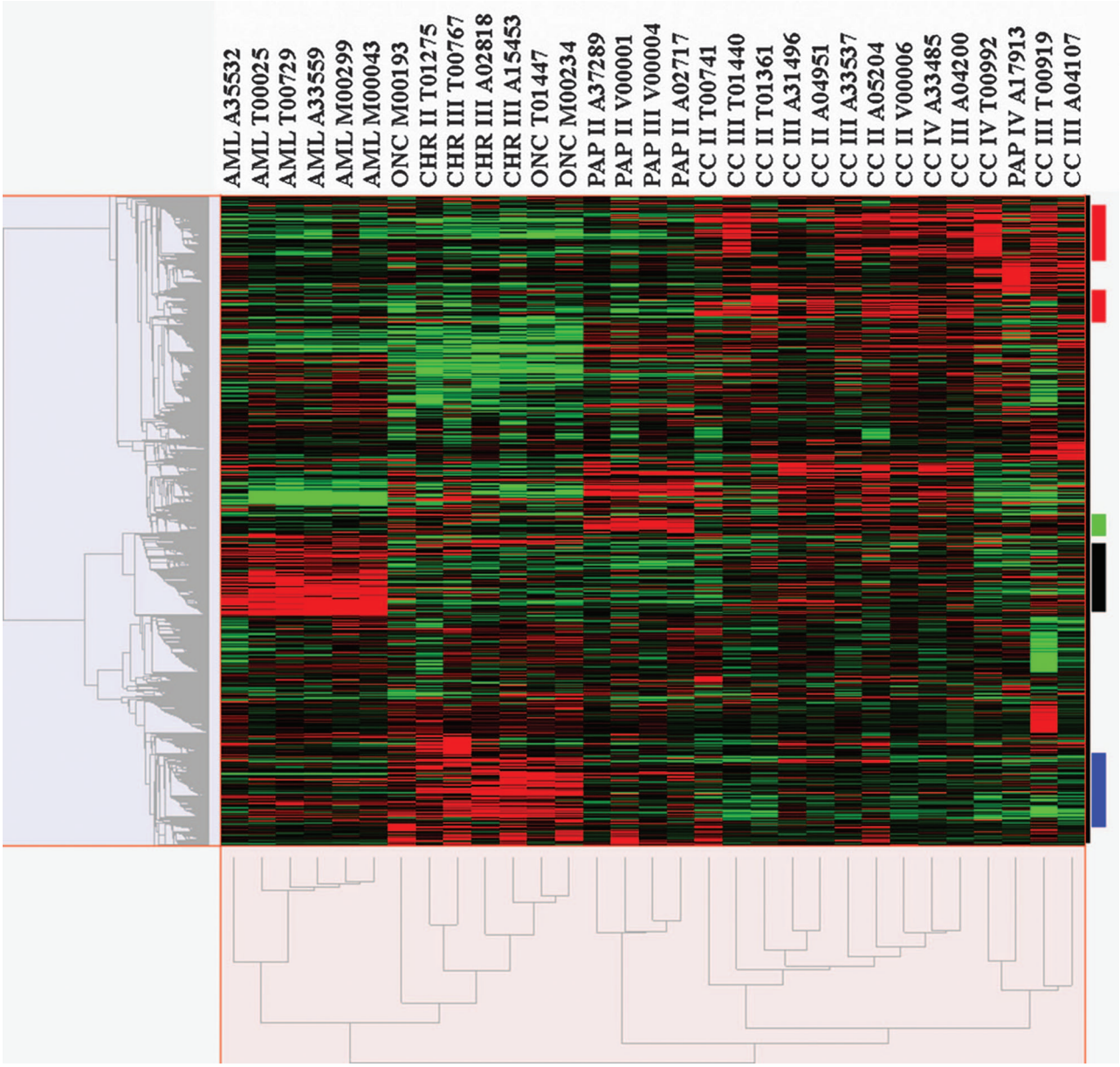

Figure 1. Unsupervised hierarchical clustering of 31 renal neoplasms. Similarity measurements are based on Pearson correlation. Median-centered differential gene expression is shown in the color-coded grid, with **columns** representing individual tumors and **rows** representing individual genes. **Red**, **green**, and **black grid blocks** indicate expression above, below, and at the median of all tumors, respectively. Based on profiles of 4030 differentially expressed genes, tumors were clustered into subgroups corresponding to clear cell RCC, papillary RCC, chromophobe RCC/renal oncocytoma, and angiomyolipoma. The sole outlier (PAP IV A17913) was a high-grade papillary RCC with sarcomatoid transformation, which was clustered with clear cell RCC. The same tumor classification was obtained when all 8746 genes on the microarray were analyzed (data not shown). **Red**, **green**, **black**, and **blue bars** to the **right** of the color-coded grid indicate genes overexpressed in clear cell RCC, papillary RCC, angiomyolipoma, and chromophobe RCC/oncocytoma, respectively. Tumor names above the grid are interpreted as follows: CC, clear cell RCC; PAP, papillary RCC; CHR, chromophobe RCC; ONC, renal oncocytoma; AML, angiomyolipoma. Roman numerals indicate Fuhrman nuclear grade. Identification codes with one letter followed by five digits are for internal tracking only. Of the carcinomas in this analysis, only one case (CC IV T00992) was documented to be metastatic.

that are difficult to distinguish by light microscopy.[13–16] The current experiments, as well as previous studies,[5,12,17–21] have confirmed that renal tumor subtypes can be classified diagnostically using microarrays, on the basis of distinct and reproducible gene expression profiles. Of 31 cases analyzed in the current study, the sole outlier was a papillary RCC with extensive sarcomatoid transformation, which was most similar to clear cell RCC in terms of gene expression. Extensive review of this case by light microscopy revealed no clear cell histology, leading us to suspect that misclassification occurred due to gene expression from the spindle cell component. Alternatively, the tumor may have been a transformed, high-grade clear cell RCC with sarcomatoid and papillary features, containing scant residual clear cell histology, which was misdiagnosed by light microscopy.

The current study represents a progression of our previous microarray analysis of renal tumors,[17] with several important enhancements. For example, the current study characterized gene expression in a larger number of

Table 2. Oxidative Phosphorylation and Energy Pathway Genes Overexpressed in Chromophobe Renal Cell Carcinoma and Renal Oncocytoma

Symbol	UniGene	GenBank	Name
Gene ontology: electron transport (GO:0006118; $P = 4.0 \times 10^{-14}$)			
ACAD8	Hs.14791	BC001964	Acyl-coenzyme A dehydrogenase family, member 8
ACADM	Hs.79158	NM_000016	Acyl-coenzyme A dehydrogenase, C-4 to C-12 straight chain
ACADSB	Hs.81934	NM_001609	Acyl-coenzyme A dehydrogenase, short
COVA1	Hs.155185	S72904	Cytosolic ovarian carcinoma antigen 1
COX5A	Hs.323834	NM_004255	Cytochrome c oxidase subunit Va precursor
COX5B	Hs.1342	AI557312	Cytochrome c oxidase subunit Vb precursor
COX7A2L	Hs.30888	NM_004718	Cytochrome c oxidase subunit VIIa polypeptide 2 like
COX7B	Hs.432170	NM_001866	Cytochrome c oxidase subunit VIIb precursor
COX8A	Hs.433901	NM_004074	Cytochrome c oxidase subunit VIII
CYC1	Hs.289271	NM_001916	Cytochrome c-1
CYP11B2	Hs.184927	X54741	Cytochrome P450, subfamily XIB polypeptide 2 precursor
CYP26A1	Hs.150595	NM_000783	Cytochrome P450, family 26, subfamily A, polypeptide 1 isoform 1
CYP2D6	Hs.333497	NM_000106	Cytochrome P450, subfamily IID, polypeptide 6
DLD	Hs.74635	J03620	Dihydrolipoamide dehydrogenase precursor
ETFDH	Hs.323468	NM_004453	Electron-transferring-flavoprotein dehydrogenase
FMO4	Hs.2664	NM_002022	Flavin-containing monooxygenase 4
GSR	Hs.193974	NM_000637	Glutathione reductase
HCCS	Hs.211571	AI801013	Holocytochrome c synthase (cytochrome c heme-lyase)
HSPC051	Hs.284292	NM_013387	Ubiquinol-cytochrome c reductase complex 7.2 kd
IVD	Hs.374536	AK022777	Isovaleryl coenzyme A dehydrogenase
MRPS30	Hs.28555	NM_016640	Mitochondrial ribosomal protein S30
NDUFB2	Hs.198272	NM_004546	NADH dehydrogenase (ubiquinone) 1β subcomplex, 2, 8 kd
NDUFB5	Hs.19236	NM_002492	NADH dehydrogenase (ubiquinone) 1β subcomplex, 5, 16 kd
NDUFS1	Hs.8248	NM_005006	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kd
NDUFS4	Hs.10758	BC005270	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18 kd
NDUFV2	Hs.51299	NM_021074	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24 kd
NOX5	Hs.160199	NM_024505	NADPH oxidase, EF hand calcium-binding domain 5
OSBP2	Hs.7740	NM_030758	Oxysterol binding protein 2
PDCD8	Hs.18720	NM_004208	Programmed cell death 8 isoform 1
QF-C	Hs.3709	NM_014402	Low molecular mass ubiquinone-binding protein
SDHB	Hs.64	NM_003000	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
SDHD	Hs.168289	NM_003002	Succinate dehydrogenase complex, subunit D precursor
UQCRC1	Hs.119251	NM_003365	Ubiquinol-cytochrome c reductase core protein I
UQCRFS1	Hs.3712	BC000649	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
Gene ontology: oxidative phosphorylation (GO:0006119; $P = 5.9 \times 10^{-9}$)			
ATP5B	Hs.406510	NM_001686	ATP synthase, H+ transporting, mitochondrial F1 complex, β
ATP5G1	Hs.80986	AL080089	ATP synthase, H+ transporting, mitochondrial F0, subunit c, isoform 1
ATP5G3	Hs.429	NM_001689	ATP synthase, H+ transporting, mitochondrial F0, subunit c, isoform 3
ATP5O	Hs.433960	NM_001697	ATP synthase, H+ transporting, mitochondrial F1, O subunit
ATP6V0B	Hs.7476	BC005876	ATPase, H+ transporting, lysosomal 21kD, V0 subunit c″
ATP6V1A1	Hs.281866	AF113129	ATPase, H+ transporting, lysosomal 70 kd, V1 subunit A, isoform 1
ATP6V1B1	Hs.64173	NM_001692	ATPase, H+ transporting, lysosomal 56
ATP6V1C1	Hs.86905	NM_001695	ATPase, H+ transporting, lysosomal 42 kd, V1 subunit C, isoform 1
ATP6V1D	Hs.272630	AF077614	ATPase, H+ transporting, lysosomal 34 kd, V1 subunit D
ATP6V1E1	Hs.77805	BC004443	ATPase, H+ transporting, lysosomal 31 kd, V1 subunit E isoform 1
Gene ontology: energy pathways (GO:0006091; $P = 5.9 \times 10^{-9}$)			
ACO2	Hs.300463	NM_001098	Aconitase 2
BPGM	Hs.198365	NM_001724	2,3-Bisphosphoglycerate mutase
CKMT2	Hs.80691	NM_001825	Sarcomeric mitochondrial creatine kinase precursor
ENO3	Hs.118804	NM_001976	Enolase 3
GCGR	Hs.208	U03469	Glucagon receptor
IDH3A	Hs.250616	AI826060	Isocitrate dehydrogenase 3 (NAD+) α precursor
NDUFA10	Hs.198271	NM_004544	NADH dehydrogenase (ubiquinone) 1α subcomplex, 10, 42kDa
OGDH	Hs.168669	NM_002541	Oxoglutarate (α-ketoglutarate) dehydrogenase (lipoamide)
OXCT1	Hs.177584	NM_000436	3-Oxoacid CoA transferase precursor
PDHA1	Hs.1023	NM_000284	Pyruvate dehydrogenase (lipoamide) α1
PDHB	Hs.979	AL117618	Pyruvate dehydrogenase (lipoamide) β
PFKM	Hs.75160	U24183	Phosphofructokinase, muscle
PHKA1	Hs.2393	NM_002637	Phosphorylase kinase, α1 (muscle)
PPARA	Hs.998	BC000052	Peroxisome proliferative activated receptor, α
SLC25A4	Hs.2043	NM_001151	Solute carrier family 25, member 4
SUCLA2	Hs.182217	NM_003850	Succinate-CoA ligase, ADP-forming, β subunit
SUCLG1	Hs.7043	NM_003849	Succinate-CoA ligase, GDP-forming, α subunit

The significance analysis of microarrays (SAM) identified genes overexpressed in chromophobe RCC/oncocytoma versus all other tumors. The GOstat program identified gene ontology functional annotation terms that were statistically overrepresented in the chromophobe RCC/oncocytoma expression profile.

Table 3. Protease Inhibitor and Extracellular Matrix Genes Overexpressed in Papillary Renal Cell Carcinoma

Symbol	UniGene	GenBank	Name
Gene ontology: enzyme inhibitor activity (GO:0004857; $P > 0.05$)			
ANXA3	Hs.1378	M63310	Annexin A3
GNAI1	Hs.203862	AL049933	G protein, α inhibiting activity polypeptide 1
SERPINE2	Hs.21858	AL541302	Plasminogen activator inhibitor type 1, member 2
SLPI	Hs.251754	NM_003064	Secretory leukocyte protease inhibitor precursor
TFPI	Hs.170279	J03225	Tissue factor pathway inhibitor 1
TFPI2	Hs.295944	AL574096	Tissue factor pathway inhibitor 2
Gene ontology: extracellular matrix (GO:0006119; $P > 0.05$)			
C6	Hs.1282	J05064	Complement component 6 precursor
FLRT3	Hs.41296	NM_013281	Fibronectin leucine rich transmembrane protein 3
GLRB	Hs.32973	AF094754	Glycine receptor β
LAMB1	Hs.82124	NM_002291	Laminin, β1 precursor
LAMC2	Hs.54451	NM_005562	Laminin, γ2 isoform a precursor
MMP15	Hs.80343	NM_002428	Matrix metalloproteinase 15 preproprotein

The significance analysis of microarrays (SAM) identified genes overexpressed in papillary RCC versus all other tumors. The GOstat program did not identify gene ontology functional annotation terms that were statistically overrepresented in the papillary RCC expression profile.

tumors (31 versus 7 cases), and included samples of papillary RCC and angiomyolipoma, in addition to clear cell RCC, chromophobe RCC, and oncocytoma. Furthermore, the current experiments, which used oligonucleotide microarrays, produced expression data from a greater number of genes than our previous assays, which used cDNA microarrays. These differences in study design could explain why the current experiments identified many more candidate expression markers for renal tumor subtypes. Nevertheless, results of the two studies were consistent, in that the current analysis confirmed many specific markers for clear cell RCC and chromophobe RCC/oncocytoma identified in our previous work.

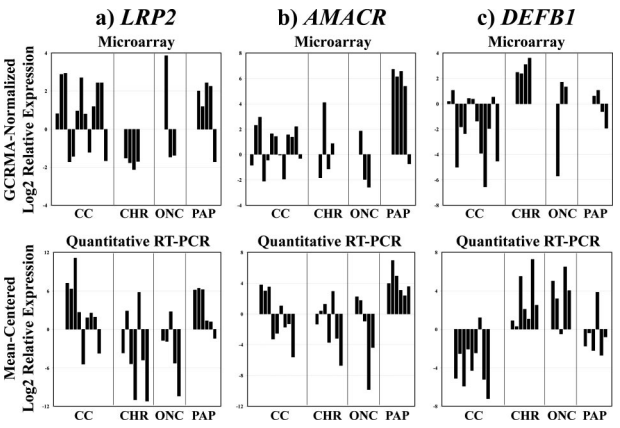

Figure 2. Differential expression of proximal and distal nephron markers in renal epithelial neoplasms: validation of microarray data by quantitative RT-PCR. Graphs at the **top** of the figure show normalized $\log_2$ gene expression ratios in tumor RNA relative to reference RNA, determined by oligonucleotide microarray. Graphs at the **bottom** of the figure show mean-centered $\log_2$ gene expression ratios in tumor RNA relative to reference RNA, determined by quantitative RT-PCR. In all graphs, **bars** indicate relative gene expression in individual tumors. Tumor subtypes are indicated below the *x* axes: CC, clear cell RCC; PAP, papillary RCC; CHR, chromophobe RCC; ONC, renal oncocytoma. **a:** Megalin/low-density lipoprotein-related protein 2 (*LRP2*, **left**). The proximal nephron marker *LRP2* was typically expressed at high levels in clear cell and papillary RCC, although the confirmatory data did not reach statistical significance ($P = 0.30$ by quantitative RT-PCR). **b:** α-Methylacyl CoA racemase (*AMACR*, **center**). The proximal nephron marker *AMACR* was overexpressed significantly in papillary RCC ($P = 0.0047$ by quantitative RT-PCR). **c:** β-Defensin 1 (*DEFB1*, **right**). The distal nephron marker *DEFB1* was overexpressed significantly in chromophobe RCC and oncocytoma ($P = 0.024$ by quantitative RT-PCR).

In the current study, clear cell RCC overexpressed several genes encoding proximal nephron markers, including *CBLN* and *LRP2* (functional partners in receptor-mediated endocytosis),[35,36] consistent with histogenetic models that relate this tumor subtype to proximal nephron epithelium.[37] Clear cell RCC also overexpressed angiogenic factors and receptors, consistent with the anastomosing vascular network that makes this tumor a promising target for anti-angiogenic therapies.[6] Angiogenesis in clear cell RCC is due primarily to loss-of-function mutation of the von Hippel-Lindau (*vHL*) gene on chromosome 3p25, the most common genetic defect in both hereditary and sporadic lesions.[38] In our data, *vHL* gene expression did not vary significantly among clear cell tumors or between renal tumor subtypes. Although cytogenetic studies were not available to correlate with the expression data, a lack of significant variation in *vHL* expression has been noted in previous microarray studies of renal tumors.[20] Clear cell tumors overexpressed a variety of immune response genes, including several members of the class II MHC. Class II MHC expression has been related to lymphocyte infiltration in RCC,[39] possibly indicative of anti-tumor immunity, and clinical studies have shown that immunomodulatory therapies are more effective against advanced clear cell RCC than cases with nonclear cell histology.[33] Thus, expression profiles of immune response genes may be therapeutically significant in the characterization of clear cell renal tumors.

Gene expression was remarkably similar in chromophobe RCC and renal oncocytoma, consistent with other microarray studies.[17,20,21] These tumor subtypes share several clinical, morphological, and molecular features,[40–43] and both arise frequently in Birt-Hogg-Dubé syndrome, an autosomal dominant, multiorgan system tumor syndrome mapped to chromosome 17p12-q11.2 (other renal tumor subtypes, including clear cell RCC, arise less frequently in this syndrome).[44,45] The Birt-Hogg-Dubé gene, termed folliculin (*FLCN*), is of unknown function and was not probed by the microarrays used in our study, precluding interpretation of our data in context of this sequence. However, recent studies have shown

Table 4. Muscle Development, Lipid Biosynthesis, and Pigmentation Genes Overexpressed in Angiomyolipoma

Symbol	UniGene	GenBank	Name
Gene ontology: muscle development (GO:00075177; $P = 3.6 \times 10^{-4}$)			
ACTA1	Hs.1288	NM_001100	α1 actin precursor
ACTA2	Hs.195851	NM_001613	α2 actin
ACTG2	Hs.378774	NM_001615	Actin, γ2 propeptide
AEBP1	Hs.439463	NM_001129	Adipocyte enhancer binding protein 1 precursor
APEG1	Hs.21639	NM_005876	Aortic preferentially expressed gene 1
CALD1	Hs.325474	AL577531	Caldesmon 1 isoform 3
COL6A3	Hs.80988	NM_004369	α3 type VI collagen isoform 1 precursor
DMD	Hs.169470	NM_004010	Dystrophin Dp427c isoform
GATA6	Hs.50924	D87811	GATA binding protein 6
HDAC9	Hs.116753	NM_014707	Histone deacetylase 9 isoform 3
ITGB1BP2	Hs.109999	NM_012278	Melusin
LAMA2	Hs.75279	NM_000426	Laminin α2 subunit precursor
MYH11	Hs.78344	AI889739	Smooth muscle myosin heavy chain 11 isoform SM1
MYL9	Hs.9615	NM_006097	Myosin regulatory light polypeptide 9 isoform a
TAGLN	Hs.433399	NM_003186	Transgelin
TPM1	Hs.77899	Z24727	Tropomyosin 1 α
TPM2	Hs.300772	NM_003289	Tropomyosin 2 β
TPM4	Hs.250641	AI214061	Tropomyosin 4
Gene ontology: lipid biosynthesis (GO:0008610; $P = 4.5 \times 10^{-3}$)			
ACACA	Hs.7232	BE855983	Acetyl-coenzyme A carboxylase α
CYP51A1	Hs.226213	NM_000786	Cytochrome P450, family 51
FADS1	Hs.132898	AL512760	Fatty acid desaturase 1
FADS2	Hs.184641	NM_004265	Fatty acid desaturase 2
FDFT1	Hs.48876	BC003573	Farnesyl-diphosphate farnesyltransferase 1
ISYNA1	Hs.405873	AL137749	Myo-inositol 1-phosphate synthase A1
LSS	Hs.93199	AW084510	Lanosterol synthase
SR-BP1	Hs.24447	NM_005866	Type I σ receptor isoform 1
PBX1	Hs.155691	NM_002585	Pre-B-cell leukemia transcription factor 1
PC	Hs.89890	NM_022172	Pyruvate carboxylase precursor
PTGDS	Hs.430637	NM_000954	Prostaglandin D2 synthase 21 kd (brain)
RODH	Hs.11958	U89281	3-Hydroxysteroid epimerase
SC4MOL	Hs.239926	AV704962	Sterol-C4-methyl oxidase-like
SC5DL	Hs.288031	D85181	Sterol-C5-desaturase-like
SCD	Hs.119597	AB032261	Stearoyl-CoA desaturase (δ-9-desaturase)
SIAT8A	Hs.82527	L32867	Sialyltransferase 8A
Gene ontology: pigmentation (GO:0048066; $P > 0.05$)			
OA1	Hs.74124	NM_000273	Ocular albinism 1 (Nettleship-Falls) protein
SILV	Hs.95972	U01874	Silver homolog
TYRP1	Hs.75219	NM_000550	Tyrosinase-related protein 1
UROD	Hs.78601	M14016	Uroporphyrinogen decarboxylase

The significance analysis of microarrays (SAM) identified genes overexpressed in angiomyolipoma versus all other tumors. The GOstat program identified gene ontology functional annotation terms that were statistically overrepresented in the angiomyolipoma expression profile.

that the Birt-Hogg-Dubé locus may be inactivated, either by loss of heterozygosity or promoter hypermethylation, in sporadic renal tumors of all histological subtypes.[46] Chromophobe RCC and oncocytoma are related to intercalated cells of the cortical collecting duct.[47,48] Correspondingly, these tumors overexpressed genes for distal nephron markers, such as *DEFB1* (a small cationic antimicrobial peptide),[49] *PVALB* (a calcium-binding protein),[50] and others (see complete microarray data). Overexpression of *DEFB1* was confirmed by quantitative RT-PCR. These findings were consistent with previous immunohistochemical studies.[18,51] Chromophobe RCC and oncocytoma also overexpressed genes related to energy pathways, electron transport, and oxidative phosphorylation, which may reflect the characteristically abundant mitochondria in their neoplastic cells.[42] Previous research has correlated high content of oxidative phosphorylation complexes in oncocytoma with a slow-growing, noninvasive phenotype, in comparison to clear cell RCC.[52] Chromophobe RCC and oncocytoma underexpressed activators of the nuclear factor-κB signaling cascade and genes related to apoptosis. In several types of cancer, nuclear factor-κB signal transduction has been shown to promote oncogenesis by inhibiting cell death and activating cell proliferation and angiogenesis.[53] However, the role of this signaling pathway in renal tumor development has not been studied in detail.

Notwithstanding their many similarities, chromophobe RCC and renal oncocytoma are distinct in their potential for malignant behavior, making accurate classification one of the most important, and difficult, diagnoses in renal tumor pathology. Immunohistochemical or other expression markers would be particularly useful in this setting, but none have attained widespread acceptance.[54] Our microarray experiments identified the *CLDN7* and *CLDN8* gene products (tight junction proteins expressed normally in distal nephron epithelium)[55] as candidate expression markers for chromophobe RCC. Immunohistochemistry suggested that *CLDN7* was expressed at the protein level in chromophobe RCC, and to a lesser de-

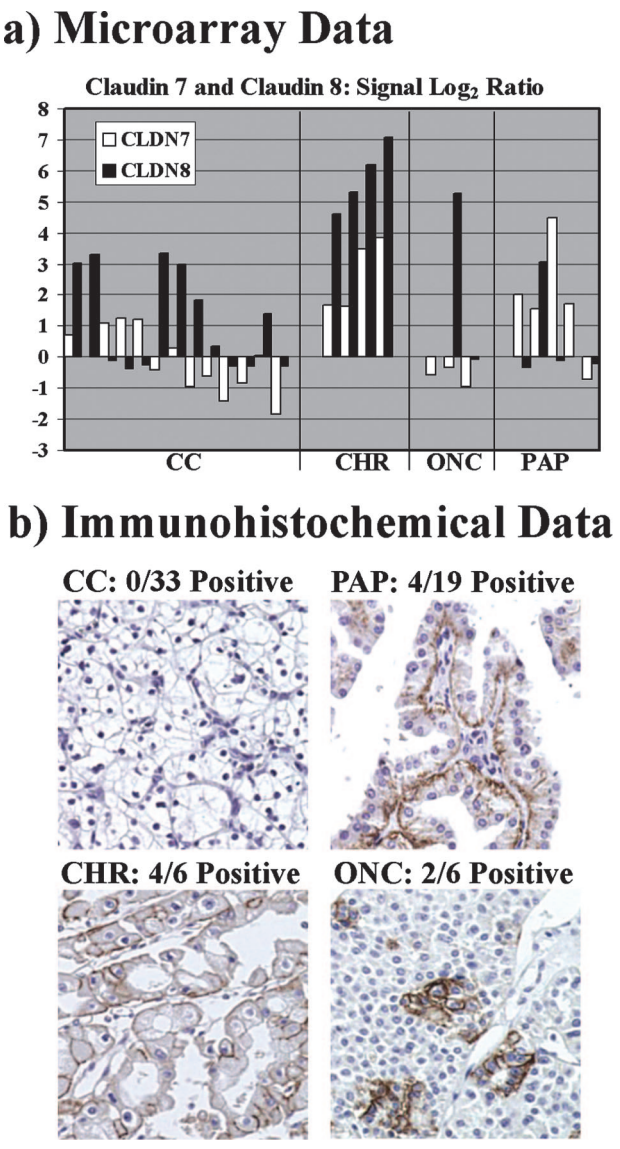

Figure 3. Expression of distal nephron claudins in renal epithelial neoplasms. **a:** Microarray data. Graph shows normalized $\log_2$ gene expression ratios in tumor RNA relative to reference RNA. **White bars**, claudin 7 (*CLDN7*); **black bars**, claudin 8 (*CLDN8*). **Bars** indicate relative gene expression in individual tumors. Tumor subtypes are indicated below the *x* axis: CC, clear cell RCC; PAP, papillary RCC; CHR, chromophobe RCC; ONC, renal oncocytoma. *CLDN7* and *CLDN8* were overexpressed in chromophobe RCC relative to oncocytoma. **b:** Immunohistochemical data. *CLDN7* gene product was detected by immunoperoxidase reactions, using diaminobenzidine (brown) as the chromogenic peroxidase substrate and hematoxylin (blue) as the nuclear counterstain. Representative images are shown for each renal tumor subtype, with tumor subtype and frequency of positive reactions indicated above each panel. *CLDN7* gene product was detected in 4 of 6 chromophobe RCCs, 2 of 6 oncocytomas, 4 of 19 papillary RCCs, and 0 of 33 clear cell RCCs. In chromophobe RCC and oncocytoma, the immunohistochemical staining pattern was membranous in tumor cells. In papillary RCC, the predominant staining pattern was cytoplasmic in stromal cells. Original magnifications, ×100.

gree in oncocytoma, but not in clear cell RCC, consistent with the mRNA data, as well as the aforementioned histogenetic models for these tumors. Larger studies are needed to validate the utility of *CLDN7* for discriminating chromophobe RCC from oncocytoma. Recently, an immunohistochemical study showed that the *RON* oncogene product (macrophage-stimulating protein receptor) was overexpressed specifically in oncocytoma,[56] although this finding was not repeated in an independent study.[57] If confirmed in future studies, *RON* and the distal nephron claudins could be clinically useful as an immunohistochemical panel with differential reactivity toward chromophobe RCC and oncocytoma.

Papillary RCC expressed several proximal nephron markers, which could indicate a relationship between this tumor and proximal nephron epithelium. In particular, papillary carcinoma strongly overexpressed the proximal nephron marker *AMACR*,[58] consistent with previous microarray experiments.[20,21] The protein product of this gene can be probed with well-established immunohistochemical assays, emphasizing its potential diagnostic utility.[59] In this study, we developed a novel quantitative RT-PCR assay for *AMACR* applicable to formalin-fixed tissues, which might complement immunohistochemistry in clinical diagnosis. Papillary carcinomas also overexpressed genes encoding serine protease inhibitors and extracellular matrix proteins. Although this expression profile has not been described before in papillary RCC, genes with this activity are known to be important factors in tumor growth and invasion, and specific gene products have been identified as potential targets of cancer therapy.[60,61] The rare, autosomal dominant familial papillary RCC syndrome is linked to oncogenic mutations of the *c-MET* gene on chromosome 7q31-34.[62] Mutations of c-*MET* are uncommon in sporadic tumors, although protein is detected in most cases by immunohistochemistry.[63,64] In our study, c-*MET* mRNA was overexpressed in several papillary carcinomas, although the difference between papillary RCC and other renal tumor subtypes did not attain statistical significance using the SAM algorithm, due to the limited number of tumors and variability in expression among cases.

This study includes one of the largest microarray analyses of renal angiomyolipoma yet performed. Angiomyolipoma is a mesenchymal neoplasm caused by proliferation of perivascular epithelioid cells. Most cases are composed of variable amounts of mature adipose, smooth muscle, and atypical thick-walled blood vessels.[65] Angiomyolipoma is associated with the autosomal dominant genetic disorder tuberous sclerosis, caused by mutations in the *TSC1* or *TSC2* tumor suppressors on chromosomes 9q34 and 16p13.[66–68] In our study, *TSC2* was underexpressed consistently in angiomyolipoma, although the difference did not reach statistical significance using the SAM algorithm. The *TSC1* and *TSC2* gene products form a heterodimer that antagonizes cell growth and angiogenesis.[69,70] Therefore, our finding of *TSC2* underexpression, as well as significant overexpression of vascular endothelial growth factors B and D (*VEGFB*, *VEGFD*), could be related to the distinctive angiogenesis of angiomyolipoma. Atypical angiogenesis is characteristic of other tuberous sclerosis-related neoplasms,[71] and future studies are indicated to determine whether this vascularity is related to differential expression of *TSC1/2*, *VEGFB*, or *VEGFD*. In addition to angiogenic factors, angiomyolipoma overexpressed myoid, adipose, and melanocytic markers, consistent with immunohistochemical profiles of this tumor.[65,72] These

results could suggest that the grossly dissected specimens used in our study contained a typical range of vascular, myoid, and adipose histology. Laser capture microdissection would be useful to isolate areas with these histological features before microarray hybridization, to determine whether gene expression profiles in angiomyo-lipoma vary with histological pattern.

The RCC cases in our study were not associated with long-term clinical follow-up, preventing discovery of expression profiles that correlate with outcome. However, Takahashi and colleagues[73] used high-density cDNA microarrays to define a gene expression profile that predicted cause-specific survival, independent of histological grade and pathological stage, in a well-characterized cohort of clear cell RCC. Similarly, Vasselli and colleagues[74] analyzed the primary tumors from a series of metastatic RCC, and identified several expression markers that correlated with survival. The oligonucleotide arrays in our study contained probes for 49 of the prognostic markers described in these two reports. Based on the expression patterns of these genes, clear cell tumors in our study could be clustered into two major categories, one of which consisted entirely of high-grade lesions (Fuhrman grade III/IV; data not shown). Additional clinical follow-up will be necessary to determine whether this classification defines cases with distinct clinical outcomes.

In summary, histopathological subtypes of renal neoplasms expressed distinct, biologically relevant molecular signatures. For example, clear cell RCC was revealed as an immunogenic and angiogenic tumor related to proximal nephron epithelium. Chromophobe RCC and oncocytoma appeared to be closely related neoplasms, overexpressing distal nephron markers and energy pathway genes, and underexpressing IκB kinase/nuclear factor-κB regulators and cell death genes. Papillary RCC expressed a distinct molecular signature, including serine protease inhibitors, extracellular matrix products, and proximal nephron markers such as *AMACR*. Angiomyolipoma was characterized as a mesenchymal tumor with adipose, smooth muscle, vascular, and melanocytic features. Additional clinical or pathological properties may be revealed by further analysis of the microarray data and the case cohort. Consistent with our previous research, microarray data could be translated into specific quantitative RT-PCR and immunohistochemical assays using formalin-fixed paraffin-embedded tissues, which may be applicable in clinical settings for diagnosis and clinical management of renal tumors.

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