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Gene Expression Patterns in Renal Cell Carcinoma Assessed by Complementary DNA Microarray

John P.T. Higgins,* Rajesh Shinghal,† Harcharan Gill,† Jeffrey H. Reese,‡ Martha Terris,§ Ronald J. Cohen,¶ Michael Fero,‖ Jonathan R. Pollack,* Matt van de Rijn,* and James D. Brooks†

From the Department of Pathology, Stanford University School of Medicine, Stanford, California; the Department of Urology,† Stanford University School of Medicine, Stanford, California; the Department of Urology,‡ Santa Clara Valley Medical Center, San Jose, California; the Department of Urology,§ Palo Alto VA Hospital, Palo Alto, California; the Department of Surgery,¶ Urological Research Centre, University of Western Australia, Perth, Western Australia, Australia; and the Department of Genetics,‖ Stanford University School of Medicine, Stanford, California*

Renal cell carcinoma comprises several histological types with different clinical behavior. Accurate pathological characterization is important in the clinical management of these tumors. We describe gene expression profiles in 41 renal tumors determined by using DNA microarrays containing 22,648 unique cDNAs representing 17,083 different UniGene Clusters, including 7230 characterized human genes. Differences in the patterns of gene expression among the different tumor types were readily apparent; hierarchical cluster analysis of the tumor samples segregated histologically distinct tumor types solely based on their gene expression patterns. Conventional renal cell carcinomas with clear cells showed a highly distinctive pattern of gene expression. Papillary carcinomas formed a tightly clustered group, as did tumors arising from the distal nephron and the normal kidney samples. Surprisingly, conventional renal cell carcinomas with granular cytoplasm were heterogeneous, and did not resemble any of the conventional carcinomas with clear cytoplasm in their pattern of gene expression. Characterization of renal cell carcinomas based on gene expression patterns provides a revised classification of these tumors and has the potential to supply significant biological and clinical insights. ***(Am J Pathol 2003, 162:925–932)***

Renal cell carcinoma comprises 3% of malignancies worldwide and is increasing in incidence.[1–8] Between one-third and one-half of the 30,000 patients diagnosed with renal cell carcinoma in the United States each year will die of their disease. Only a fraction of renal cell carcinomas are responsive to radiotherapy, immunotherapy, or chemotherapy, making the disease difficult to control once it has spread beyond the kidney. Tumor stage and surgical resectability are the most important prognostic factors for renal cell carcinoma, but the histological subtype and grade provide additional prognostic information. Renal cell carcinomas can be classified into conventional (both granular and clear cell variants), papillary, and chromophobe carcinomas based on their histological appearance. Although each of the histological variants displays a spectrum of clinical behavior, conventional carcinomas tend to behave aggressively, while chromophobe carcinomas and papillary carcinomas follow a more indolent clinical course.[9]

Each histological variant of renal cell carcinoma shows distinct karyotypic abnormalities including loss of chromosome 3p in clear cell carcinomas[10] and trisomy of chromosomes 7, 12, 16, 17, and 20 in papillary carcinomas.[11] These molecular genetic alterations have been coupled with histological features to form a revised classification of renal cell tumors.[12] Since changes in gene copy number (gene amplification, aneuploidy, and allelic loss) as well as differences in histological appearance have been associated with altered gene expression patterns, we anticipated that each of the histological subtypes of renal cell carcinoma would have unique and easily identifiable gene expression signatures. In addition, an analysis of seven renal cell carcinomas by Young et al[13] suggested that gene expression profiles could be associated with histological subtype.

To further test whether gene expression patterns can add to the classification of renal cell carcinomas, we performed DNA microarray analysis on 41 renal tumors of diverse histological types and on three normal kidney samples using 22,648 element-spotted DNA microarrays representing 17,083 different human genes. We report that gene expression patterns can readily distinguish between the histological subtypes of renal cell carcinoma and that conventional renal cell carcinomas with predominantly granular cytoplasm may represent a heteroge-

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John P.T. Higgins and Rajesh Shinghal contributed equally to this work.

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Address reprint requests to James D. Brooks, Department of Urology, Stanford University School of Medicine, Stanford, CA 94305. E-mail: jbrooks@stanford.edu.

neous group distinct from conventional clear cell carcinoma. Our data set is a potential rich source for novel markers of the subtypes of renal cell carcinoma and may offer unique biological insights into these tumors.

Materials and Methods

Tissue Samples

Samples were obtained from fresh nephrectomy specimens and immediately frozen on dry ice. Paraffin sections from each specimen were reviewed by a single pathologist (J.P.T.H.) and classified according to Union International Contre le Cancer (UICC) and American Joint Committee on Cancer (AJCC) criteria[14] and can be reviewed at http://genome-www.stanford.edu/renal_cell_carcinoma/. Our series included 28 conventional renal cell carcinomas (three of which had accompanying renal vein tumor thrombi), four papillary carcinomas, three chromophobe carcinomas, two oncocytomas, and one angiomyolipoma. The conventional carcinomas were subdivided into 23 clear cell and five granular cell carcinomas based on whether they contained cytoplasmic granules in the majority of the tumor cells. Three normal kidney samples were obtained from areas of the nephrectomy specimens uninvolved by carcinoma.

Microarray Analysis

Methods for mRNA extraction, hybridization to 17,083 gene (represented by 22,648 cDNAs) DNA microarrays, and interpretation of data have been described elsewhere[15–18] and detailed protocols are available at http://cmgm.Stanford.EDU/pbrown/. Each tumor mRNA was hybridized against a common reference pool of mRNA made from 11 different cell lines as we have described previously.[17]

Average linkage hierarchical cluster analysis was used to organize gene expression data. Expression levels for each transcript were centered across all samples. We selected transcripts whose expression level differed by a factor of four or greater from the normalized mean level of expression in at least two of the samples. We further restricted our analysis to those genes with a fluorescent hybridization signal greater than 100 over background in 80% of the experimental samples. Of the initial list of 22,648 cDNAs, 1550 cDNAs met these criteria.

The significance analysis of microarrays (SAM) procedure was used to identify genes significantly associated with the conventional granular cell carcinoma subtype.[19] SAM computes a two-sample T-statistic for the normalized log ratios of the gene expression levels for each gene. It thresholds the T-statistics to produce a "significant" gene list and provides an estimate of the false discovery rate (genes that differ between samples by chance alone) from randomly permuted data. SAM also allows identification of genes associated with classes of tumors that may not be evident by hierarchical clustering analysis.

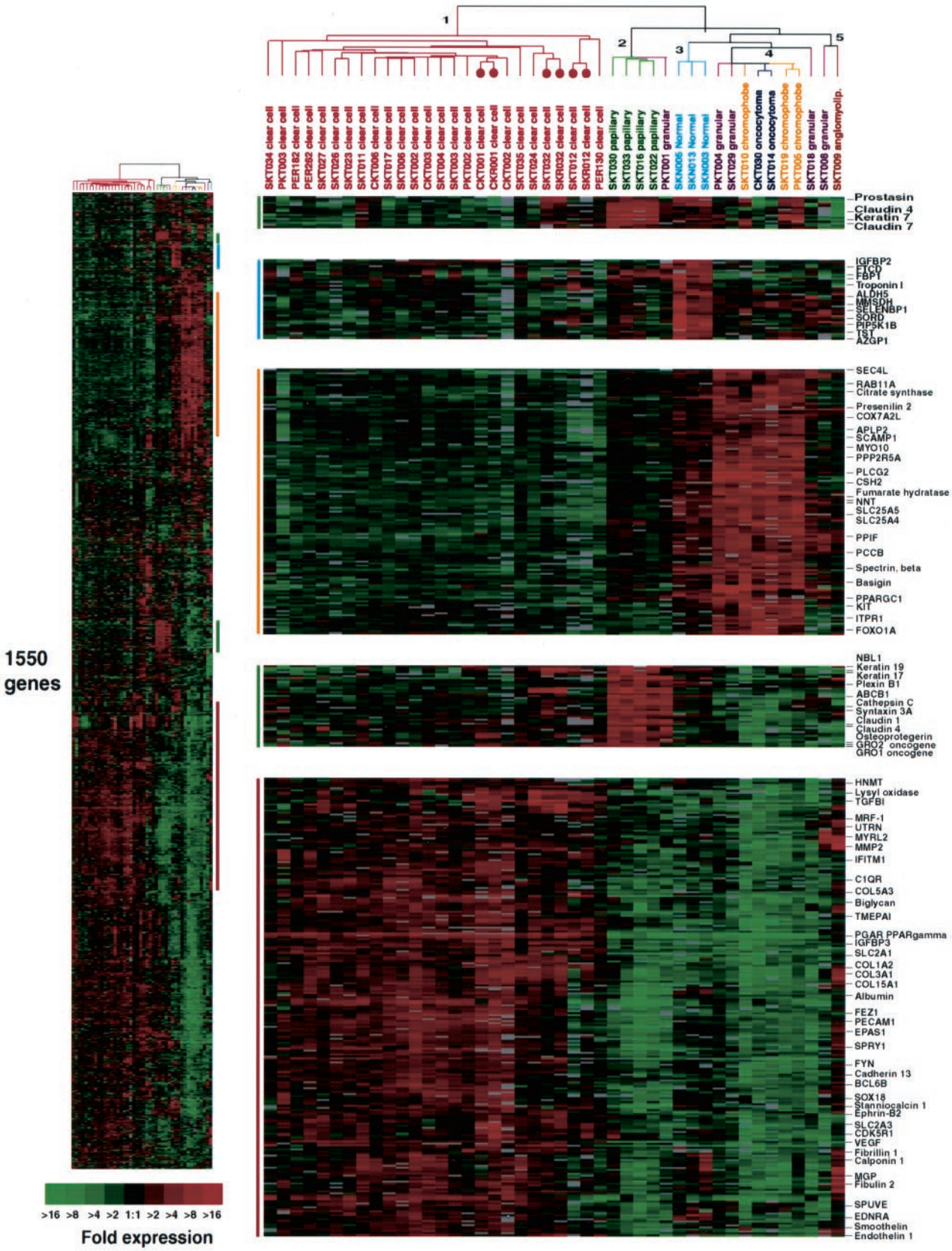
Immunohistochemistry

Paraffin blocks were available from 30 of the 38 cases. Four-μm sections were cut and immunohistochemistry was performed using monoclonal antibodies for CK7 (clone OV-TL 12/30, BioGenex, San Ramon, CA; 1:200), CD10 (clone 56C6, Novocastra, Newcastle-on-Tyne, UK; 1:50), and vimentin (clone V9, BioGenex; ready to use) according to previously published protocols.[20]

Results

We used 22,648 element-spotted DNA microarrays to characterize the gene expression patterns in 41 renal tumors and three normal kidney samples. We included renal tumors of diverse histology including conventional clear cell, conventional granular cell, papillary, and chromophobe carcinomas as well as two oncocytomas and an angiomyolipoma. Hierarchical cluster analysis was used to group samples, based on the degree of similarity of their gene expression profiles, as well as genes, based on their pattern of expression across all of the samples (Figure 1 and on-line at http://genome_www.Stanford.edu/renal_cell_carcinoma). We selected a set of 1550 transcripts with significant variation over the samples measured (fourfold from the mean) that were well measured above background in at least 80% of the samples. Expression profiles over this set of transcripts separated the tumors into two broad classes: one consisting entirely of conventional clear cell carcinomas and the other of the remaining tumor subtypes and normal kidney samples. Three samples harvested from renal vein thrombi displayed expression patterns highly similar to their respective primary tumors, suggesting that each tumor has a unique and characteristic gene expression pattern (Figure 1).

Figure 1. Hierarchical clustering analysis for all genes across all arrays. A representation of the variation in expression of 1550 genes in the 38 tumors and three normal samples is shown at the **left**. The **right panel** depicts the detailed sample dendrogram in which three primary tumors and their associated renal vein/vena cava thrombi are designated on the dendrogram by branches with **filled circles** at the ends. Each row represents a single gene. Each column represents a single sample. The hierarchical clustering algorithm organizes the experimental samples only on the basis of overall similarity in their gene expression. **Green squares** indicate transcript levels below the mean; **black squares**, transcript levels equal to the mean; **red squares**, transcript levels greater than the mean; **gray squares**, technically inadequate or missing data. Gene filtering criteria were for fourfold variation from the normalized mean and at least 80% well measured spots. **Colored bars** adjacent to the clustering table indicate the position of the enlarged images. The color of the bar indicates the type of sample in which the cluster of genes is expressed. **Green**, papillary gene cluster; **blue**, normal gene cluster; **orange**, chromophobe-oncocytoma gene cluster; **red**, conventional clear cell carcinoma gene cluster. Selected gene names are shown.


1
2
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SKT034 clear cell
PKT003 clear cell
PER182 clear cell
PER262 clear cell
SKT027 clear cell
SKT026 clear cell
SKT023 clear cell
SKT011 clear cell
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SKT032 clear cell
SKR032 clear cell
SKT012 clear cell
SKR012 clear cell
PER130 clear cell
SKT030 papillary
SKT033 papillary
SKT016 papillary
SKT022 papillary
PKT001 granular
SKN005 Normal
SKN013 Normal
SKN003 Normal
PKT004 granular
SKT029 granular
SKT010 chromophobe
CKT030 oncocytoma
SKT014 oncocytoma
SKT019 chromophobe
PKT005 chromophobe
SKT018 granular
SKT008 granular
SKT009 angiomyolip.
Prostasin
Claudin 4
Keratin 7
Claudin 7
IGFBP2
FTCD
FBP1
Troponin I
ALDH5
MMSDH
SELENBP1
SORD
PIP5K1B
TST
AZGP1
SEC4L
RAB11A
Citrate synthase
Presenilin 2
COX7A2L
APLP2
SCAMP1
MYO10
PPP2R5A
PLCG2
CSH2
Fumarate hydratase
NNT
SLC25A5
SLC25A4
PPIF
PCCB
Spectrin, beta
Basigin
PPARGC1
KIT
ITPR1
FOXO1A
NBL1
Keratin 19
Keratin 17
Plexin B1
ABCB1
Cathepsin C
Syntaxin 3A
Claudin 1
Claudin 4
Osteoprotegerin
GRO2 oncogene
GRO1 oncogene
HNMT
Lysyl oxidase
TGFBI
MRF-1
UTRN
MYRL2
MMP2
IFITM1
C1QR
COL5A3
Biglycan
TMEPAI
PGAR PPARgamma
IGFBP3
SLC2A1
COL1A2
COL3A1
COL15A1
Albumin
FEZ1
PECAM1
EPAS1
SPRY1
FYN
Cadherin 13
BCL6B
SOX18
Stanniocalcin 1
Ephrin-B2
SLC2A3
CDK5R1
VEGF
Fibrillin 1
Calponin 1
MGP
Fibulin 2
SPUVE
EDNRA
Smoothelin
Endothelin 1
1550 genes
>16 >8 >4 >2 1:1 >2 >4 >8 >16
Fold expression

Table 1. Results of Immunohistochemistry

Tumor type	CD10	Vimentin	Keratin 7
Angiomyolipoma (n = 1)	neg	str	neg
Chromophobe (n = 3)	1str	1wk, 2neg	2str, 1wk
Conventional/clear (n = 15)	7str, 6wk, 1neg	12str, 3wk	1str, 2wk, 12neg
Conventional/granular (n = 4)	2str, 2wk	2str, 2wk	4wk
Oncocytoma (n = 1)	str	wk	wk
Papillary (n = 3)	3neg	2str, 1wk	3str

Str, strong staining; wk, weak staining; neg, no staining.

Like the conventional carcinomas with clear cytoplasm, the papillary carcinomas showed expression patterns highly similar to each other, as did all of the normal kidney samples. The gene expression patterns of the remaining tumor samples were somewhat more diverse. The two oncocytomas and three chromophobe carcinomas showed strikingly similar patterns over the 1550 transcripts, as reflected in the short branch length of the dendrogram connecting these tumors. Interestingly, cases of conventional renal cell carcinoma in which the majority of the tumor cells show granular cytoplasm did not group together. One sample showed an expression pattern that was most similar to the papillary carcinomas and three others resembled the chromophobe/oncocytoma group (Figure 1). One tumor had a highly distinctive expression pattern and did not group with any other carcinoma, but clustered instead with the angiomyolipoma. The histology of the conventional carcinomas with granular cytoplasm was re-reviewed in light of the expression profiles and in no case did they meet diagnostic criteria for any other histological category. Histology for all samples analyzed can be viewed at the supplemental website: http://genome-www.stanford.edu/renal_cell_carcinoma/.

Inspection of the genes that characterize each of the histological subtypes of renal cell carcinoma offers some insights into the biology of these tumors. A group of 230 transcripts was overexpressed in all conventional carcinomas with clear cytoplasm, most of which are unnamed or poorly characterized (Figure 1). *VEGF*, the glucose transporters 1 and 3 (*SLC2A1* and *SLC2A3*),[21] *endothelin-1*,[22,23] and *insulin-like growth factor binding protein 3*[23] were all expressed at high levels in the clear cell carcinomas and are all primarily regulated by the HIF-1 transcription factor. Normally, HIF-1 levels are regulated by the von Hippel-Lindau (*VHL*) protein, which targets HIF-1 protein for ubiquitylation-mediated degradation.[24] The *VHL* gene is inactivated in most conventional clear cell carcinomas, and HIF-1 protein is expressed at high levels. In our data set, neither *VHL* nor *HIF-1* transcript levels varied across the tumor samples. Since the *VHL* gene is usually inactivated by loss of one allele and mutation of the second, and HIF-1 levels are regulated post-translationally, it is not surprising that neither gene shows altered transcript levels. Interestingly, *HIF2-α* (*EPAS1*) was highly expressed in the clear cell carcinomas. Although less well characterized than HIF1-α, EPAS1 is thought to be regulated by VHL,[25] and our data suggests it may be regulated transcriptionally. *Collagen types I, III, IV, V, and VI, lysyl oxidase, heparan sulfate proteoglycan 2*, and *fibronectin* may be expressed by cells in the interstitium, while *PECAM1* (*CD31*), *EPAS1*, *VEGF receptor 2*, and *cadherin 5* are known to be expressed in endothelial cells and probably reflect the rich vascularity of these tumors.

Genes overexpressed in papillary carcinoma consist of two distinct clusters of 15 and 44 genes (Figure 1). These genes include cytokeratin subsets that appear to be uniquely overexpressed in these tumors and could be exploited diagnostically. Papillary carcinomas also express high levels of *α-methylacyl-CoA racemase* (*AMACR*), a gene found to be overexpressed in prostate cancer by DNA microarray analysis.[26,27] The oncogenes *GRO 1* and *GRO 2* are also expressed at high levels in these tumors, although it is unclear whether they are expressed by the malignant cells or by the characteristic foamy macrophages that line the papillae in these tumors. Additionally, other transcripts, such as osteoprotegerin and I factor, are known to be expressed by macrophages.

Chromophobe carcinomas and oncocytomas both show increased expression of the stem cell factor receptor (*KIT*, *CD117*), a gene not previously implicated in these neoplasms (Figure 1). This gene is also highly expressed in gastrointestinal stromal sarcoma (GIST) by immunohistochemistry[28] and by DNA microarray analysis[29] and is a potential therapeutic target.[30] Several genes that are highly expressed in oncocytomas and chromophobe carcinomas, including *nicotinamide nucleotide transhydrogenase*, *fumarate hydratase*, and *solute carrier family 25 members 4&5*, encode mitochondrial proteins. Oncocytomas are known to be rich in mitochondria,[31] and chromophobe carcinomas contain abundant microvesicles that likely represent altered mitochondria.[32,33]

The gene expression profiles of conventional carcinomas with granular cytoplasm are distinct from those with clear cytoplasm and appeared heterogeneous. Since their expression patterns over the 1550 transcripts were highly diverse and shared similarities with the other histological subtypes of renal malignancies, we were not able to identify a discrete set of genes that characterize these malignancies using hierarchical clustering analysis. We used the SAM procedure to compare the gene expression patterns of the conventional granular renal carcinomas to the other renal tumors in our data set to identify genes that characterize the granular cell phenotype. Although SAM analysis identified 91 transcripts highly expressed in the granular cell carcinomas and two expressed at significantly lower levels compared to the other tumors, none of these 93 transcripts showed consistent expression changes across all of the conventional

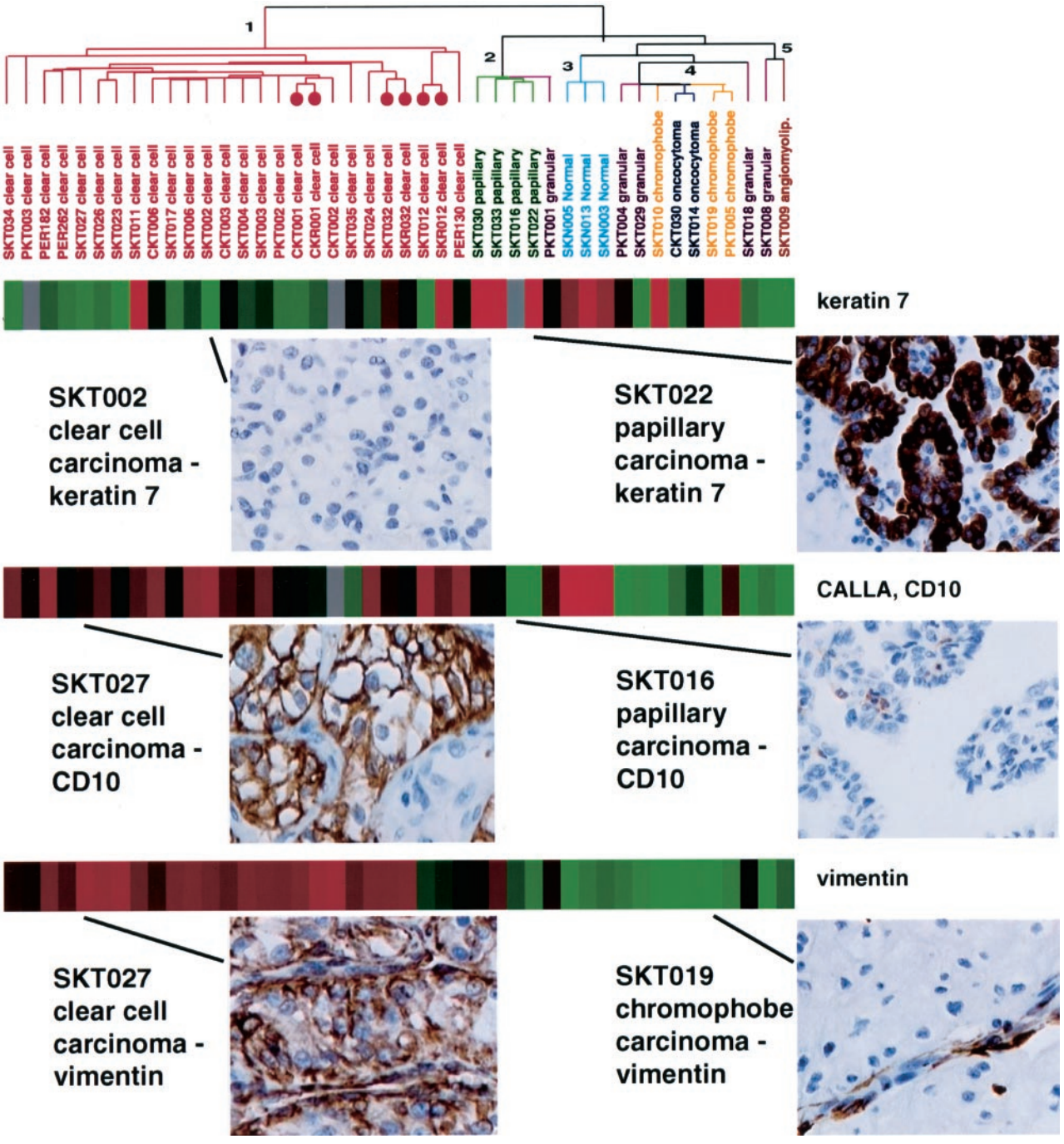

Figure 2. Immunohistochemical analysis of CK7, CD10, and vimentin and correlated mRNA expression data. mRNA expression data for the keratin 7, CD10, and vimentin genes, displayed using the same color key used in Figure 1. Immunohistochemical stains for the proteins encoded by these genes are shown for selected tumors below the corresponding mRNA expression results.

carcinomas with granular cytoplasm, nor did they reliably distinguish these tumors from other histological subtypes (data not shown).

To evaluate whether the transcripts we identified could help distinguish between tumor subtypes, we selected commercially available antibodies for three proteins whose transcripts were differentially expressed (keratin 7, CD10, and vimentin) and performed immunohistochemical analysis of paraffin-embedded tissues from the set of tumors that were analyzed by DNA microarray (Table 1). In each case, the protein expression pattern usually reflected the RNA expression pattern observed in the DNA microarray analysis (Figure 2). Most notably, vimentin readily distinguished between the clear cell carcinoma and chromophobe carcinoma subtypes, a distinction that may be difficult on hematoxylin and eosin-stained sections. Thus, these data sets represent a potential source of immunohistochemical markers that may aid in distinguishing renal tumor subclasses.

Discussion

Classification of renal neoplasms based on gene expression largely recapitulates that based on the histological appearance. Conventional clear cell carcinomas showed expression patterns highly distinct from the other subtypes of renal cell carcinoma and from normal kidney. Papillary carcinoma and normal kidney also showed remarkably characteristic gene expression profiles. Chromophobe carcinomas shared many features of gene expression with the oncocytomas, while conventional renal carcinomas with granular cytoplasm were more heterogeneous and shared expression patterns with several histological subtypes, yet were clearly distinct from conventional clear cell carcinoma. Similar correlation between gene expression patterns and histological class has been observed in tumors from several different organ sites.[16,29,34,35]

We were surprised that conventional renal cell carcinomas with granular cytoplasm are molecularly distinct from typical conventional carcinomas with clear cytoplasm. Before standardization of renal tumor pathology by the Heidelberg classification and the Union International Contre le Cancer, many tumors designated as granular cell were actually oncocytomas, papillary carcinomas, chromophobe carcinomas, and other rare variants.[12,14] Now, only those tumors with granular cytoplasm that fail to meet criteria for any of these diagnoses are classified with clear cell carcinomas as "conventional carcinomas" because of their similar clinical behavior. Some investigators have even suggested that clear cell and granular carcinomas are variants of a single histological and molecular subclass. However, our data suggest that the histological differences between clear and granular cell carcinoma correlate with distinct differences in gene expression patterns. Perhaps more surprising, the conventional carcinomas with granular cytoplasm display significant heterogeneity in their gene expression profiles and do not appear to represent a single tumor subtype. This heterogeneity will need to be confirmed by analysis of additional tumors; however, if true, it could signal differences in the biology of these tumors and may help in identifying tumors that respond differently to therapy.

Despite their distinctive appearance histologically, oncocytomas and chromophobe carcinomas had highly similar patterns of gene expression. In part, this similarity may be due to the small number of these tumors we analyzed or the set of transcripts we used in our analysis. However, this similarity also could reflect their common histogenesis from the distal renal tubule, or similar biological behavior since both tumors are often clinically indolent.[36,37] One intriguing possibility is that these tumors arise from a common molecular genetic lesion and therefore display similar gene expression profiles. Individuals affected with the Birt-Hogg-Dubé syndrome develop multifocal chromophobe carcinomas and oncocytomas, often within the same kidney, suggesting these tumors may arise from a common genetic alteration.[38] Transcriptional profiling of additional chromophobe carcinomas and oncocytomas will be necessary to shed light on the molecular genetic similarities and differences between these tumors.

Two groups, using techniques similar to ours, have analyzed gene expression profiles of renal cell carcinoma, and reported expression profiles similar to ours. Young et al[13] analyzed seven renal carcinomas on DNA microarrays with 7075 genes and identified 32 transcripts overexpressed and 48 transcripts underexpressed in conventional compared to chromophobe renal cell carcinoma (RCC) and an oncocytoma. Forty-eight of these 80 genes were well measured in our tumors and all but three showed expression patterns identical to their report. Takahashi et al[39] identified 32 transcripts with increased expression and 77 with decreased expression in clear cell carcinoma relative to normal kidney. Eighty-five of the 89 transcripts represented in our data set showed patterns of expression identical to that seen in their clear cell cancers.[39] Using high-density DNA arrays spotted on nylon membranes, Boer et al[40] described 1738 transcripts differentially expressed between normal and cancerous renal tissues. Tumors were not characterized by histological subtype, making comparison with our results difficult. However, 123 transcripts (representing 89 unique Unigene clusters) were differentially expressed between conventional and chromophobe carcinomas. Of the 64 that were well measured on our microarrays, 53 showed expression patterns that matched those reported by Boer et al.[40] Therefore, microarray analysis of gene expression appears to be robust and reproducible despite the use of different tumor sets and measurement on different array platforms.

Expression patterns associated with papillary and granular cell carcinomas have not been described. Our data set, therefore, may serve as a rich source of molecular markers that aid in the discrimination of renal cell carcinoma subtypes. The very good correlation of keratin 7, CD10, and vimentin mRNA expression noted on microarray with protein expression seen by immunohistochemistry highlights the potential of cDNA microarray technology to identify novel diagnostic antibodies that could be used clinically.

Microarray analysis of malignancies from other organ sites has revealed molecular subtypes of tumors that are histologically indistinguishable and discrete gene expression profiles have been identified that correlate with clinical outcomes.[16,17,34,35,41–44] Takahashi et al[39] identified 51 genes with expression patterns that correlated with adverse outcome in 29 patients followed 10 years after resection of their clear cell carcinoma. When we performed hierarchical clustering analysis of the conventional clear cell carcinomas in our data set using 45 of their 51 genes that were present on our arrays, the tumors were sorted into two groups with expression patterns similar to those reported (data not shown). Additional clinical follow-up of patients in our series will be necessary to determine whether the expression patterns observed for this set of genes or additional sets of genes carry prognostic information.

Global analysis of gene expression using cDNA microarray technology offers significant opportunities to identify novel markers that discriminate between classes

of renal tumors and holds promise in identifying molecular subclasses of tumors with differing prognosis. These data provide a starting point for identification of proteins with altered expression in renal cell carcinomas. Such proteins may be measurable in the serum or urine and could serve as new markers for this disease. They could be used clinically to monitor response to therapy or, possibly, to screen those at high risk for renal cell carcinoma such as individuals with hereditary forms of the disease. Ultimately, biological insights gleaned from microarray analysis of gene expression in renal cell carcinomas may provide new targets for immune or biological therapies.

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