

Short Communication

High-Throughput Tissue Microarray Analysis to Evaluate Genes Uncovered by cDNA Microarray Screening in Renal Cell Carcinoma

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Many genes and signaling pathways are involved in renal cell carcinoma (RCC) development. However, genetic tumor markers have not gained use in RCC diagnostics and prognosis prediction. Identification and evaluation of new molecular parameters are of utmost importance in cancer research and cancer treatment. Here we present a novel approach to rapidly identify clinically relevant molecular changes in cancer. To identify genes with relevance to RCC, a cDNA array analysis was first performed on 5184 cDNA clones on a filter to screen for genes with differential expression between the renal cancer cell line CRL-1933 and normal kidney tissue. There were 89 differentially expressed genes in the cancer cell line, one of them coding for vimentin, a cytoplasmic intermediate filament. In a second step, a renal cancer tissue microarray containing 532 RCC specimen was used to determine vimentin expression by immunohistochemistry. Vimentin expression was seen frequently in clear cell (51%) and papillary RCC (61%), but rarely in chromophobe RCC (4%) and oncocytomas (12%). Furthermore, vimentin expression was significantly associated with poor patient prognosis ($P < 0.007$) independent of grade and stage. These results obtained from minute arrayed tumor samples match well with previous findings on vimentin expression in renal tumors. It is concluded that the combination of tumor arrays and cDNA arrays is a powerful approach to rapidly identify and further evaluate genes that play a role in tumor biology. (*Am J Pathol* 1999, 154:981–986)

Renal cell carcinoma (RCC) is one of the ten most frequent malignancies in Western societies. Initiation and progression of RCC is thought to be due to an accumulation of genetic changes involving numerous different genes.^{1,2} Genes potentially involved in kidney cancer include the von Hippel-Lindau gene on chromosome 3p,^{3,4} the epidermal growth factor receptor gene on 7p,^{5,6} the transforming growth factor α gene on chromosome 2p,^{7,8} and the c-myc oncogene on chromosome 8q.^{9,10} However, it is likely that many of the genes involved in the initiation and progression of renal cancer are currently unknown.

Genetic information is accumulating at a rapid pace from new genomic technologies and approaches. Up to 30,000 human genes have already been mapped accurately to the chromosomes.¹¹ Using cDNA arrays, it is now possible to perform a large-scale expression survey to identify candidate target genes.¹² However, renal cancer is a phenotypically and genetically heterogeneous group. The most common histological subtypes of RCC include clear cell (80%), papillary (about 10%), chromophobe (<5%), and collecting duct (<1%) carcinomas. Oncocytomas are benign renal tumors. Previous studies have shown that these histological subtypes are genetically and biologically different.^{2,13} The role that specific genetic alteration plays in determining prognosis, phenotype-genotype correlation, and clinical patterns needs to be investigated for each of these tumor types.¹⁴

Although cDNA arrays allow expression analysis of thousands of genes in one tumor specimen, the examination of hundreds of tumor specimens representing different tumor stages, histological grades, and histological subtypes is necessary to establish definitive frequency information for each of the emerging candidate genes. Investigation of all new cancer genes requires consider-

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able investment of time and resources. Our recently developed tumor tissue microarray method permits rapid molecular profiling of hundreds of cancer specimens¹⁵ (high-through-put analysis) and is therefore ideally suited for further analyses of candidate genes emerging from cDNA array experiments.

In this study we first used cDNA arrays to identify genes that play a role in RCC and then analyzed one candidate gene on a tumor array for its potential clinical significance. The results show that the combination of tumor arrays and cDNA arrays is a powerful approach to rapid identification and further evaluation of genes that play a role in RCC biology.

Materials and Methods

cDNA Array Experiment

cDNA was synthesized and radioactive labeled using 50 μ g total RNA from normal kidney (Invitrogen) and a renal cancer cell line (CRL-1933) (American Type Culture Collection, Manassas, VA) according to standardized protocols (Research Genetics, Huntsville, AL). Release I of the human GeneFilters (Research Genetics) was used for differential expression screening. A single membrane contains 5184 spots, each representing 5 ng of cDNA of known genes or expressed sequence tags (EST). After separate hybridization the two cDNA array filters (Research Genetics) were exposed to a high resolution screen (Canberra Packard, Zürich, Switzerland) for three days. The gene expression pattern of 5184 genes in normal tissue and the tumor cell line was analyzed and compared on a phosphor imager (Cyclone, Packard). Signals that genes/ESTs were absent or present on one of the two filters were identified visually.

Tumors

For the construction of the renal tumor microarray block, our collection of 615 renal tumors after nephrectomy was screened for availability of representative paraffin-embedded tissue specimens. Tumor specimens from 532 renal tumors and tissue from 6 normal kidneys were available for the tumor array. The tumors were staged according to TNM classification,¹⁶ graded according to Thoenes,¹⁷ and histologically subtyped according to the recommendations of the UICC¹⁴ by one pathologist (HM). Core tissue biopsies 0.6 mm in diameter were taken from carefully selected morphologically representative regions of individual paraffin-embedded renal tumors (donor blocks) and precisely arrayed into a new recipient paraffin block (45 mm \times 20 mm) using a custom-built instrument.¹⁵ Sections of the resulting tumor tissue microarray block 5 μ m thick were transferred to glass slides using the paraffin sectioning aid system (adhesive coated slides PSA-CS4x, adhesive tape, UV lamp, Instrumedics Inc., Hackensack, NJ) to support the cohesion of 0.6-mm array elements.

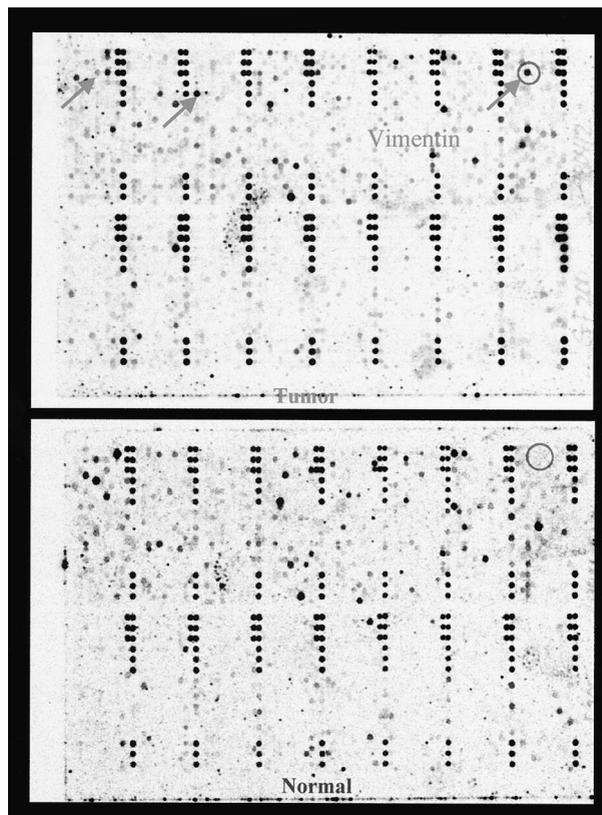


Figure 1. cDNA array: examples of overexpressed genes in the renal cancer cell line (CRL-1933) are indicated by arrows. The dot for the vimentin gene is circled.

Immunohistochemistry

Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite, Vectra Laboratories, Geneva, Switzerland). A monoclonal antibody was used for vimentin detection (anti-vimentin, Boehringer Mannheim, Mannheim, Germany, 1:160). Tumors were considered positive for vimentin if an unequivocal cytoplasmic positivity was seen in tumor cells. Vimentin positivity in endothelial cells served as an internal control. The vimentin status of epithelial cells was defined as negative (no staining) or positive (any cytoplasmic staining).

Clinical Data

Overall survival data were obtained by reviewing the hospital records, by direct communication with the attending physicians, and from the Cancer Registry of Basel. Patients were evaluated from the time of biopsy diagnosis to the last known follow-up. Clinical follow-up data were available for 386 patients.

Statistics

Contingency table analysis was used to analyze the relationship between vimentin expression, grade, stage, and tumor type. Overall survival was defined as the time between nephrectomy and patient death. Survival rates were plotted using the Kaplan-Meier method. Survival

differences between the groups were determined with the log-rank test. A Cox proportional hazard analysis was used to test for independent prognostic information.

Results

cDNA Array

Figure 1 shows the result obtained by hybridizing two cDNA array membranes with radioactive-labeled cDNA from normal kidney and from tumor cell line CRL-1933. The experiment resulted in 89 differentially expressed genes/ESTs. Overexpression in CRL-1933 was found for 38 sequences including 26 named genes and 12 ESTs, whereas 51 sequences (25 named genes, 26 ESTs) were not expressed in the cell line. The sequence of one of the up-regulated genes in the cell line was identical to that of vimentin.

Vimentin Expression Tested on Renal Tumor Array

The presence of epithelial tumor cells was tested for every tissue cylinder using a hematoxylin and eosin (H&E)-stained slide. Representative tumors are shown in Figure 2, A-C. Vimentin expression could be evaluated on the tissue cylinders in 483 tumors and all 6 normal kidney tissues. Examples of vimentin-positive and -negative tumors are shown in Figure 2D. Vimentin expression was more frequent in clear-cell, papillary, and collecting duct carcinomas than in chromophobe RCC and oncocytoma. Expression of vimentin was detected in 194 of 383 clear cell RCC (51%), 35 of 57 papillary RCC (61%), 1 of 23 chromophobe RCC (4%), and 2 of 3 collecting duct RCC (66%) cases. Only 2 of 17 oncocytomas showed a weak vimentin expression (12%). Normal renal tubules did not express vimentin. The association between vimentin expression and histological grade and tumor stage was evaluated only for clear cell RCC (Table 1). Vimentin expression was more frequent in grade II (44%) and grade III (42%) than in grade I (13%) RCC ($P < 0.0001$). Vimentin expression was more common in higher tumor stages (40% in stage pT1/2 versus 60% in stage pT3/4), but this difference was not significant ($P = 0.09$).

Clinical Outcome and Vimentin Expression

The median follow-up was 37 months (range, 1–241 months). Poor overall survival was strongly related to high histological grade ($P < 0.0001$) and advanced tumor stage ($P < 0.0001$). The association between patient prognosis and vimentin expression was evaluated for patients with clear cell RCC (Figure 3). Vimentin expression was strongly associated with short overall survival ($P = 0.007$). Multivariate proportional hazards analysis indicated that vimentin expression was a predictor of poor prognosis independent of stage and grade. The relative risk for vimentin was 1.6 ($P = 0.01$; Table 2) in clear cell RCC.

Discussion

The results of this study indicate the power of combining cDNA arrays and tissue microarrays for the identification of novel genes in cancer and rapidly exploring their clinical significance.

cDNA arrays were first used to search for genes that were differentially expressed in kidney cancer compared to normal kidney tissue. A kidney cancer cell line was selected for that purpose to assure large quantities of high-purity tumor RNA. The finding of more than 80 differentially expressed genes in the cell line CRL-1933 not only highlights the complexity of molecular changes that are present in these cells, but also demonstrates the fact that high-throughput expression surveys such as cDNA arrays generate novel experimental bottlenecks. Evaluation of the dozens or hundreds of candidate genes emerging from one cDNA experiment on a representative set of uncultured primary tumors would take years if traditional methods of molecular pathology were used. Our recently developed tumor tissue microarray technology was designed to facilitate such studies.¹⁵ Tissue arrays allow the simultaneous *in situ* analysis of hundreds of tumors on the DNA, RNA, and protein levels. During this study we constructed a kidney cancer tissue array consisting of 532 renal tumors, 386 of which had clinical follow-up data available.

Vimentin is a cytoplasmic intermediate filament (molecular weight 57000) which is characteristic of mesenchymal cells, but usually not expressed in epithelial cells. Early studies described the presence of vimentin in epithelial cells *in vitro*¹⁸ and suggested that it was induced by *in vitro* cultivation. To investigate the usefulness of our kidney tumor array for the analysis of candidate genes emerging from cDNA experiments, vimentin was selected for four reasons: high-level overexpression in the cancer cell line; previous reports of a prognostic significance of vimentin expression in breast and cervical cancer;^{19,20} availability of previous data on the prevalence of vimentin positivity in RCC subtypes, allowing a validation of the array results by comparison with results from the literature;^{21–23} and availability of an antibody suitable for analysis of formalin fixed tissues. This last reason was particularly important because antibodies for gene products that are found to be differentially expressed in cDNA arrays are often not available or not applicable to formalin-fixed tissues. Accordingly, it would greatly improve the value of tissue arrays for the analysis of novel genes if RNA *in situ* hybridization could reliably be performed on formalin-fixed tissue arrays.

If tumor arrays are used to investigate prevalence or prognostic significance of molecular changes, the critical issue is the extent to which minute tissue samples are representative of their donor tumors. The findings of this study suggest that significant results can be obtained on tumor arrays. Expression of vimentin has previously been described in 30% to 53% of formalin-fixed RCC specimens.^{21,22,24} The prevalence on arrayed samples (50%) was in the same range. In addition, differences in the vimentin expression between renal tumor subtypes were confirmed. Vimentin was detected frequently in papillary

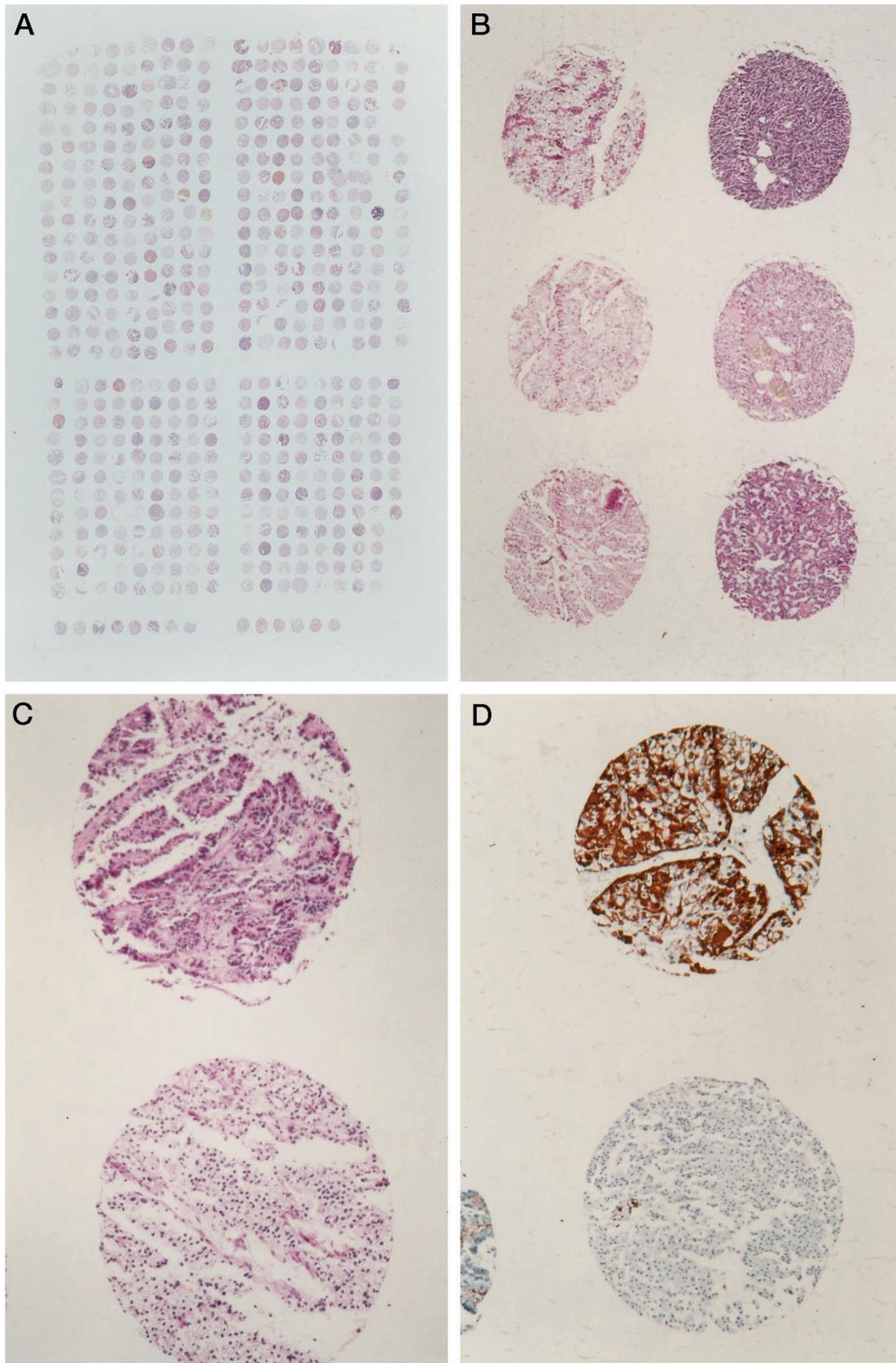


Figure 2. A: Overview of the renal tumor array with 532 tissue cylinders (H&E; original magnification, $\times 4.2$). B: Renal tumor array. Morphology of 6 different renal tumors (H&E; original magnification, $\times 42$). C: Two different renal tumor subtypes, papillary RCC (top) and clear cell RCC (bottom). (H&E; original magnification, $\times 140$). D: Immunohistochemistry: clear cell RCC with cytoplasmic vimentin expression (top) and chromophobe RCC without vimentin expression (bottom) (ABC-Elite method, anti-vimentin; original magnification, $\times 88$).

Table 1. Vimentin Expression and Tumor Phenotype in Clear Cell RCC (*n* = 383)

Vimentin expression	Grade			Stage*	
	G1	G2	G3	pT1/2	pT3/4
Number (%) of positive tumors	26 (13)	86 (44)	82 (42)	76 (40)	115 (60)
<i>P</i> -value	<0.0001			0.09	

*Three tumors were excluded because pT stage could not be defined retrospectively.

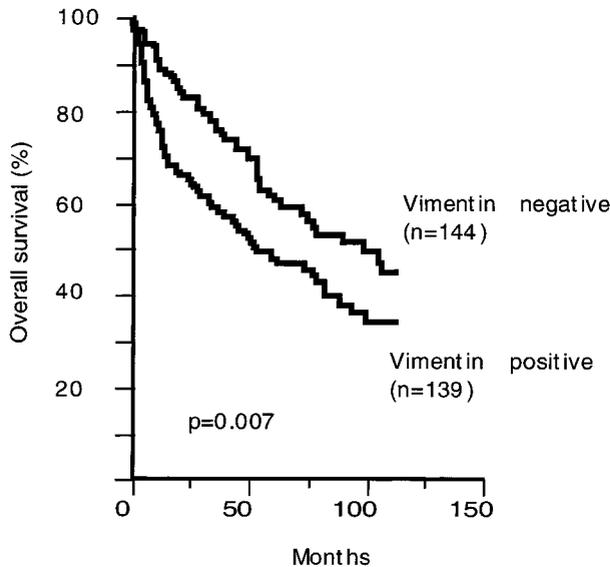


Figure 3. Overall survival analysis for clear cell RCC with and without vimentin expression (log-rank test).

and clear cell RCC but rarely in oncocytoma and chromophobe RCC as previously described.^{23,25,26} A higher progression rate in vimentin-positive than in vimentin-negative tumors was previously suggested in a study involving 93 RCC.²⁷ More than 300 RCC with clinical follow-up information were analyzed on our tumor array. The results not only confirmed a prognostic relevance of vimentin expression in RCC; multivariate statistical analysis further suggested that vimentin may have independent prognostic significance in clear cell RCC. These data provide further evidence that results obtained on arrays can be highly congruent with findings obtained from analysis of much larger tissue specimens. In earlier studies, the same gene amplification frequencies were found on a breast cancer array as had previously been reported for *c-myc*, *erbB-2*, *cyclin D1*, and *20q13* in breast cancer.¹⁵ Also, the analysis of *c-myc*, *erbB-2*, and *cyclin D1* amplifications on an array composed of 397 samples from 17 different tumor types had shown a high

Table 2. Proportional Hazard Analysis of Clear Cell RCC

Variables	Relative risk (Confidence intervals)	<i>P</i> -value
pT 1/2 versus pT 3/4	2.0 (1.4–3.2)	0.0004
Histologic grade	1.7 (1.2–2.3)	0.0006
Vimentin expression	1.6 (1.1–2.4)	0.01

level of agreement with the previous literature (P. Schraml, unpublished findings).

Taken together, the available data suggest that tumor tissue arrays may clearly facilitate the translation of findings from basic research into clinical applications. Novel molecular markers emerging from high-throughput expression surveys could first be analyzed on tumor arrays containing large numbers of tumors with clinical follow-up information on survival or response to specific therapies. Such studies can be done very rapidly. For example, sectioning, immunostaining, and analysis of a stained slide with more than 500 tumors took less than 3 days in this study. In the second step the analysis of conventional (large) diagnostic histological and cytologic specimens could then be restricted to those markers for which promising data emerged during the initial array-based analyses. Accordingly, it will now be necessary to examine vimentin expression on larger tissue specimens to confirm its independent prognostic significance in clear cell RCC. If the array data are confirmed, vimentin immunohistochemistry should be included in prospective studies investigating prognostic markers in RCC.

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