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*Original Article*

# Microarray gene expression profiling using core biopsies of renal neoplasia

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**Abstract**: We investigate the feasibility of using microarray gene expression profiling technology to analyze core biopsies of renal tumors for classification of tumor histology. Core biopsies were obtained ex-vivo from 7 renal tumors—comprised of four histological subtypes—following radical nephrectomy using 18-gauge biopsy needles. RNA was isolated from these samples and, in the case of biopsy samples, amplified by in vitro transcription. Microarray analysis was then used to quantify the mRNA expression patterns in these samples relative to non-diseased renal tissue mRNA. Genes with significant variation across all non-biopsy tumor samples were identified, and the relationship between tumor and biopsy samples in terms of expression levels of these genes was then quantified in terms of Euclidean distance, and visualized by complete linkage clustering. Final pathologic assessment of kidney tumors demonstrated clear cell renal cell carcinoma (4), oncocytoma (1), angiomyolipoma (1) and adrenalcortical carcinoma (1). Five of the seven biopsy samples were most similar in terms of gene expression to the resected tumors from which they were derived in terms of Euclidean distance. All seven biopsies were assigned to the correct histological class by hierarchical clustering. We demonstrate the feasibility of gene expression profiling of core biopsies of renal tumors to classify tumor histology.

**Key words:** cDNA microarray, gene expression profiling, biopsy, amplification, kidney tumor

## Introduction

There has been an increase in the detection of incidental small renal masses (SRM), due to the widespread use of abdominal radiographic imaging [1-4]. A considerable portion of these masses are benign or indolent and do not necessarily require surgery. Radiologic separation of benign from malignant renal lesions is not possible in most cases [1, 5], opening the possibility of over- or under-treatment. Renal masses can be classified into different tumor subtypes, each with different molecular features and prognosis [6, 7]. Treatment options for renal masses include surgical resection, ablation, observation, and systemic therapy. Biopsy of renal masses to determine malignancy, tumor histology, and prognosis may be of increasing relevance in deciding which of these treatments is most appropriate.

Renal biopsy has traditionally had a limited role in the clinical management of renal tumors, due to concerns of inaccuracy and needle tract seeding. More recent reports demonstrate renal biopsies to be safe and accurate [1, 8-14], with the ability to influence

clinical management in up to 34-41% of patients [8, 10, 15].

Using microarray technology to analyze renal tumors, the expression of thousands of genes can be monitored concurrently. Gene expression profiles have been identified that can be used to classify renal tumors into different histologic and prognostic groups [16-21]. The small amounts of RNA contained in biopsy tissue is amenable to amplification and subsequent microarray analysis [22]. Gene expression profiles from biopsy tissues have been used to classify other tumors, such as breast cancer [23-27].

In this pilot study, we assess the feasibility of performing microarray analysis of core biopsies of renal tumors in order to classify tumor histology.

## Materials and Methods

*Tissue Collection*

Core biopsies of 7 renal tumors were performed ex-vivo on radical nephrectomy specimens using an 18-gauge biopsy needle. Biopsy samples were immediately snap frozen in liquid nitrogen for RNA extraction. Tissue was also obtained from each parent tumor and corresponding normal tissue and immediately snap frozen in liquid nitrogen.

*Tissue processing and preparation of RNA*

Total RNA was isolated from the frozen tissue samples using TRIzol reagent with 200μg RNase-free glycogen (Invitrogen, Carlsbad, CA) and purified either by precipitation with 2.5M LiCl2 (Ambion Inc., Austin, TX), or through a Qiagen RNeasy column (QIAGEN Inc., Valencia, CA). The purity and integrity of the RNA was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Rockland, DE) and by the presence of distinct 18S and 28S bands following RNA gel electrophoresis. The extracted RNA was amplified using a MessageAmp aRNA Kit (Ambion Inc.) to produce amplified RNA. RNA from 6 normal specimens from non-cancerous tissue was extracted, purified, and pooled as a normal reference for the study.

cDNA Microarray Construction and Procedure
Microarray slides spotted with 19,968 cDNA clones from the Research Genetics 40K Human Clone Set (Research Genetics Inc., Huntsville, AL) were constructed at the Van Andel Research Institute. A total of 20-50 μg of total RNA from the tumor and an equal quantity of pooled normal total RNA were reverse transcribed using Superscript II (Invitrogen) and oligo-d(T)20VN primer (Invitrogen) in the presence of Cy5-dCTP or Cy3-dCTP (PerkinElmer Life and Analytical Sciences, Boston, MA). A total of 2 μg of aRNA from the tumor biopsies and 25 μg of pooled normal total RNA from matched normal samples were reverse transcribed using Superscript II (Invitrogen) and random primers (Invitrogen) for biopsy samples or oligo-d(T)20VN (Invitrogen) for pooled normal specimens in the presence of Cy5-dCTP or Cy3-dCTP (PerkinElmer Life and Analytical Sciences). Following direct labeling, the two cDNA probes were purified using a PCR Purification Kit (Qiagen) and hybridized for 20 hours at 50○C to a microarray slide. The slides were washed, dried, and scanned at 532nm and 635nm using a Scan Array Lite (PerkinElmer Life and Analytical Sciences, Boston, MA).

*Data Analysis*

Microarray data were analyzed with GenePix software version 5.0 (Axon Instruments, Inc., Foster City, CA) and data files were normalized using pin-tip dependent loess normalization. After normalization, genes were filtered for presence (> 50% of samples) and variation (standard deviation > 2.0) in the tumor (i.e. non-biopsy) samples. Missing values in the resulting data set were imputed using K-nearest neighbors imputation. To identify the relationship in terms of gene expression between tumor and biopsy samples, the Euclidean distance was calculated between each pair of samples. These relationships were then visualized using hierarchical clustering based on complete linkage. All data processing and analysis was performed using the R statistical analysis framework.

## Results

Seven tumor samples and corresponding ex-vivo biopsy samples—corresponding to 4 distinct tumor subtypes—were analyzed by microarray gene expression profiling. The samples include four renal cell carcinomas, 1 oncocytoma, 1 angiomyolipoma, and one

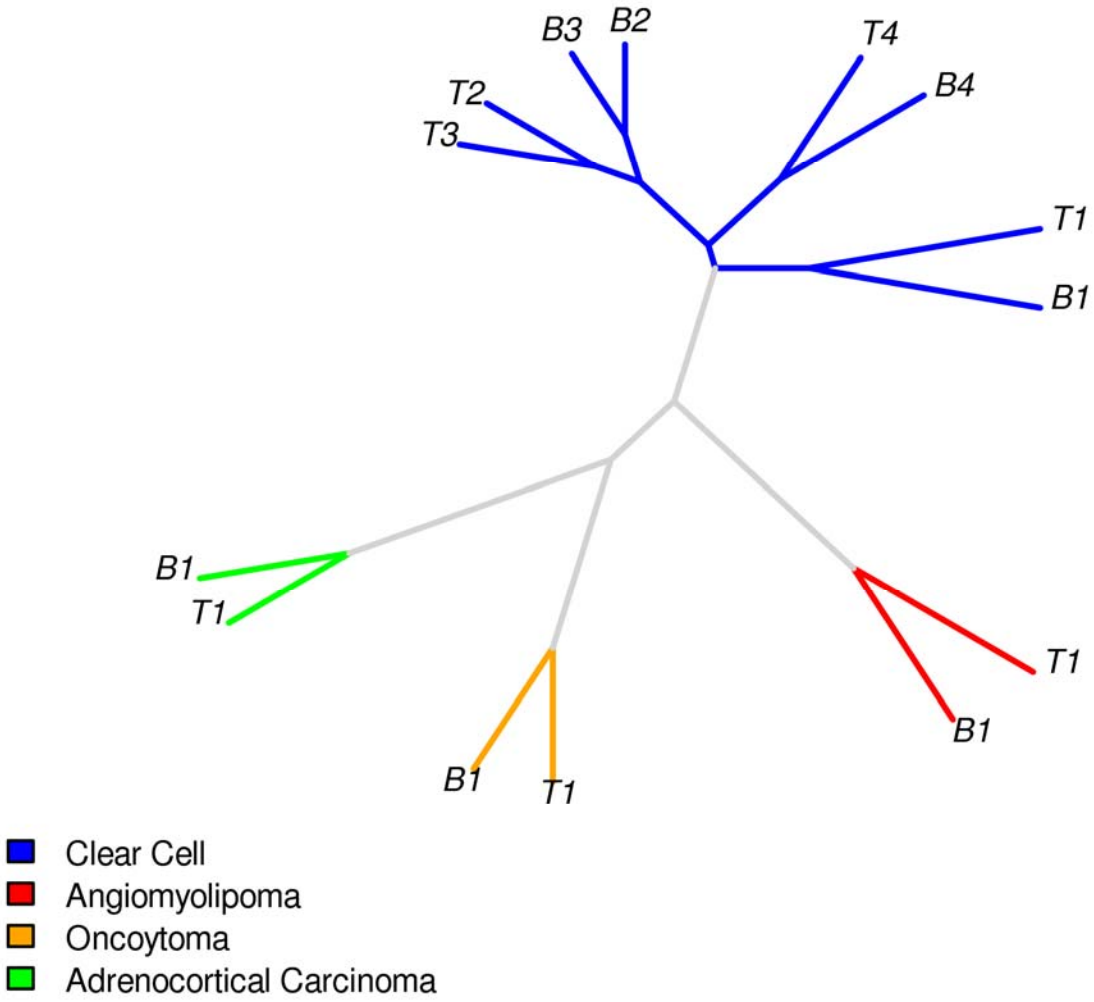


**Figure 1.** Unsupervised hierarchical cluster analysis of 7 corresponding biopsy and tumor sample pairs using filtered gene set demonstrates that all of the tumor/biopsy sample pairs have closely related gene expression profiles. Branches of the dendrogram are colored according to histological subtype. T=tumor, B=Biopsy, number indicates sample pair to which each sample belongs. Length of the branch is proportional to Euclidean distance between samples.

adrenocortical tumor included as an anatomically relevant control. When examined by hierarchical clustering based on genes that varied significantly between the various tumor samples, all 7 biopsy samples clustered with the tumor(s) of the corresponding histological subtype (**Figure 1**). Indeed, 5 of the 7 biopsy samples clustered most closely to the tumor sample from which they were taken.

Interestingly, the stringent filtering criteria we applied to the data resulted in a very small classifier. Only 48 genes were required for the classification shown. Less stringent filtering (e.g., standard deviation across non-biopsy samples of < 1.0) produced larger gene sets that exhibited similar sample clustering.

To determine how correlated the biopsy and tumor samples were to each other, we next calculated the correlation matrix for these samples (**Figure 2**). This analysis indicated that samples of a given histological type were highly correlated to each other (generally, Pearson's $r > 0.7$). In contrast, samples from differing subtypes had low correlation.

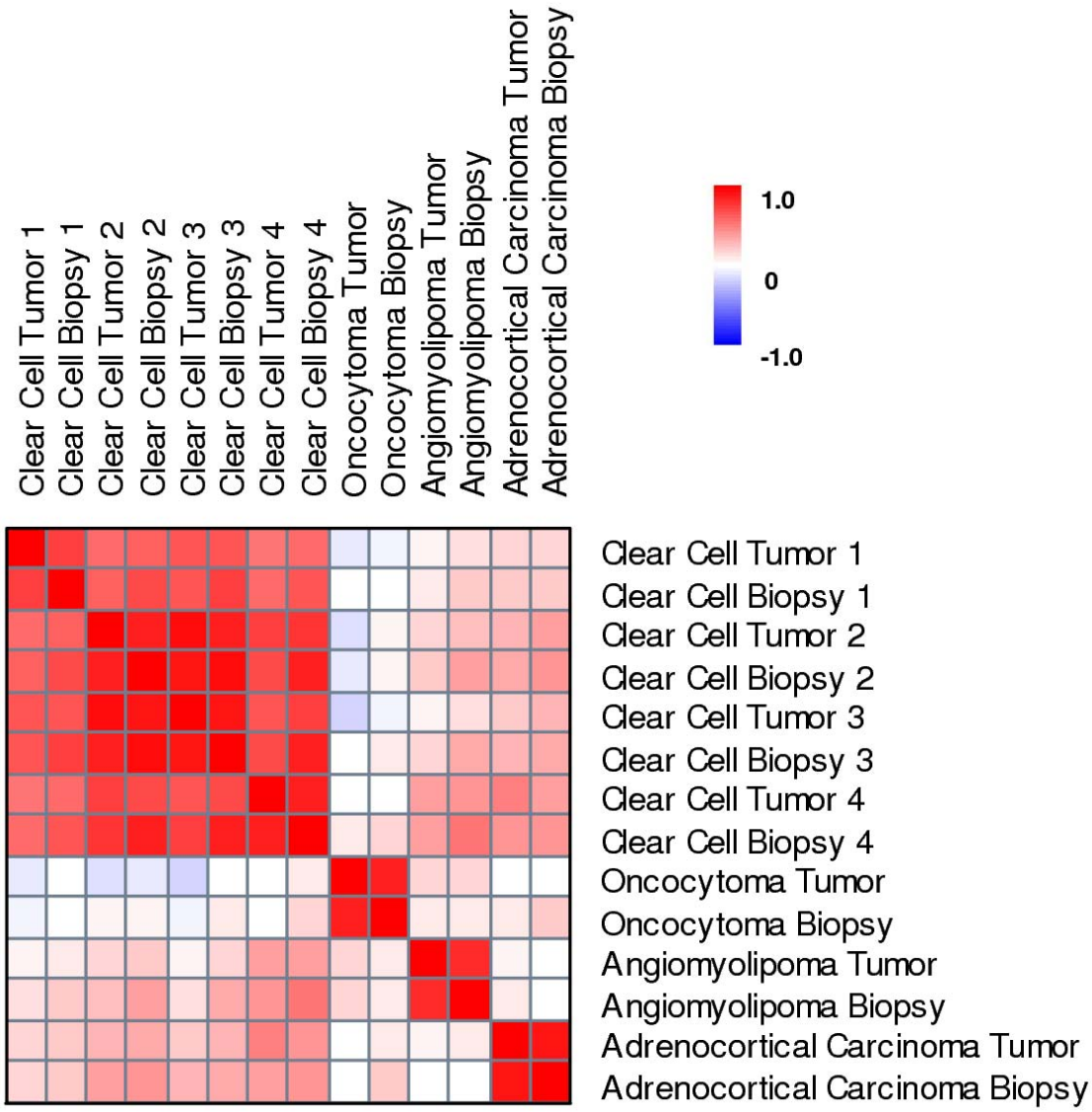


**Figure 2.** Correlation matrix between biopsy and tumor samples. Squares are color coded according to Pearson's r as the measure of correlation between samples, as indicated in the legend.

We noted that biopsy samples did not always cluster most strongly with their parent tumors; specifically, clear cell RCC biopsy samples 2 & 3 correlated more strongly with one another than with their corresponding parent tumors. The correlation matrix in **Figure 2** demonstrates that this is related to the very high correlation in gene expression between these two tumors as well as their biopsies, as opposed to lower correlation between parent tumor and biopsy. Regardless, these clear cell biopsies were correctly classified as clear cell RCC according to their expression profiles. Thus, in all cases, biopsy samples were clustered into the correct histological tumor class.

## Discussion

The number of renal masses being detected is increasing, with the greatest increase seen in tumors under 4 cm [2-4]. The histology and natural history of a renal mass is difficult to predict using only radiographic imaging [1, 5]. Renal masses can be classified into different tumor subtypes, each with different molecular

features and prognosis [6, 7]. Treatment options for renal masses include surgical resection, ablation, observation, and systemic therapy. Biopsy of renal masses, followed by molecular analysis by gene expression profiling, could potentially help determine malignancy, tumor histology and prognosis, to help guide clinical management. However, clinicians have traditionally been reluctant to rely on biopsy results to guide clinical management due to the perceived high rate of biopsy failure and inaccuracy, as well as, the theoretical risk of needle tract seeding [5, 8, 12, 28].

However, recent reports demonstrate needle biopsy of renal masses to be safe and accurate in distinguishing between malignant and benign renal tumors [1, 8-14, 29]. In the largest study of core biopsies for small renal masses, Shannon et al. examined 235 core biopsies and showed a 100% biopsy accuracy rate for distinguishing malignant from benign lesions and a 98% rate for determining histologic tumor type [10]. Other recent reports of renal core biopsies have shown a diagnostic accuracy rate of 90-100% [8, 11-13, 29] and a nondiagnostic rate of 0-20% [10, 30]. A core renal biopsy using a 16 or 18 gauge needle has replaced fine needle aspiration because it provides better characterization of benign and malignant pathology, and a lower frequency of insufficient samples [31]. Results of needle biopsy of renal tumors influenced clinical management in 34-41% of patients [8, 10, 15]. The perceived risk of needle tract seeding appears to be unfounded and renal biopsy has not been shown to increase oncologic risk of cancer progression or recurrence [32].

Gene expression profiling, performed on tissue from surgical specimens, has been used to differentiate between different subtypes of kidney tumors [16-21]. Each subtype of RCC has its own distinct molecular signature [16, 20, 21]. However, microarray analysis of core needle biopsy samples of kidney tumors has not been performed. Barocas et al. demonstrated that core biopsies of renal masses, performed ex-vivo after surgical resection, provided adequate material for molecular analysis by fluorescent in situ hybridization (FISH) and real time polymerase chain reaction (RT-PCR) analysis, and that the addition of molecular analysis to the histopathologic interpretation resulted in a 7-12% improvement in diagnostic accuracy [33, 34].

Our study demonstrates the feasibility of using amplified RNA from renal core biopsy tissue for cDNA microarray analysis. Gene expression profiles correctly predicted histologic subtype of all renal core biopsies and parent tumor samples. To our knowledge, this study is the first to demonstrate the feasibility of microarray analysis of biopsy samples to classify renal tumors by histologic subtype.

Although all clear cell RCC biopsies clustered together under an RCC signature, we noted that two cases of clear cell biopsies clustered more strongly with one another than with their parent tumors. We speculate that the RNA amplification process performed from biopsy samples may slightly shift expression profiles from the original tumor. Because all clear cell RCC samples underwent similar RNA amplification, they may have come to resemble each other more closely than their parent tumors. However, these samples still show high correlation of expression profiles with their parent tumors, and are still appropriately classified as clear cell RCC by expression profiling. Thus, although RNA amplification may subtly change the gene expression ratios of biopsy samples, this amplification process does not affect classification of biopsy samples into appropriate tumor class.

The fact the biopsies studied could be correctly classified using a very small classifier gene signature (48 genes) suggests that in the future it may be possible to develop a clinical assay using low- to medium-throughput technology such as quantitative RT-PCR. Such an approach would reduce the expense and labor involved in applying gene expression profiling to the task of histological classification of biopsy samples.

Our results suggest that microarray analysis of kidney biopsies may be a valuable adjunct to pathologic diagnosis of renal masses. The use of renal biopsy of renal masses with subsequent molecular analysis of biopsy tissue by microarray analysis could potentially identify patients with benign or indolent renal tumors, thus avoiding unnecessary surgical procedures on those patients. Future studies

will be required to assess the sensitivity and specificity of this approach in the context of a broader panel of tumor subtypes.

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