Repuncturing the Renal Biopsy: Strategies for Molecular Diagnosis in Nephrology

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Abstract. As has been exemplified by recent progress in the classification of cancer, future approaches to enhance the clinical diagnostic power of tissue biopsies may be based on gene expression profiles. A series of strategies to translate these approaches to the diagnosis of renal disease is here proposed. The theoretical and technical problems resulting from the small amount of starting material available from renal biopsies will be specifically addressed. A preliminary study with cDNA array–based expression data obtained from kidneys with tubulointerstitial inflammation and fibrosis suggests the feasibility of distinguishing molecular categories of renal disease. Finally, a combined conventional and molecular work-up of renal biopsies will be suggested. These approaches should add a new dimension to biopsy interpretation and provide novel information concerning renal pathogenesis, diagnosis, prognosis, and differential therapy. A coordinated effort from nephrologists and pathologists in large multicenter trials will be required to achieve this goal. It is hoped that this outlook will lead to stimulating discussions and the implementation of these innovative ideas in nephrology.

The percutaneous kidney biopsy was an enormous advance in the diagnosis, therapy, and understanding of renal disease. Since the introduction of fine needle biopsy, the histologic analysis of renal tissue has become an essential component of establishing a specific diagnosis in patients with kidney disease, a prerequisite for selective therapies. Techniques such as immunohistology and electron microscopy have allowed further refinement of the diagnostic categories. Critics of the renal biopsy have insisted on puncturing this diagnostic approach because of its lack of predictive strength and therapeutic consequence, i.e., in the management of patients with nephrosis (1). The current developments in the molecular analysis of disease processes and the study of pathologic tissue samples may, however, open new avenues in the diagnosis and management of disease. The identification of specific components of the glomerular filter, such as nephrin, podocin, and α-actinin 4, using genome-based approaches has already added a molecular component to hereditary renal disease. Incorporating the analysis of such components to that of renal biopsies represents the first step in the direction of a molecular diagnosis. The advances in the characterization of the human genome and in the technical feasibility of its expression analysis will most likely dwarf these additions to the classical investigations of renal biopsies.

Further molecular diagnostic strategies are emerging in addition to gene expression profiling. A correlation has been found between the clinical parameters in renal patients and their gene expression profiles obtained from urine and blood samples (2,3).

As the field of proteomics develops, it may also be applied to the analysis of renal biopsies paralleling the approach outlined in this review for mRNA expression (4). Strategies for genotyping inherited diseases are presently being used to differentiate acquired disease from genetic diseases (i.e., screening of early-onset FSGS for mutations in NPHS1, NPHS2, or ACTN4 [5,6]). The potential genotyping of individuals for single nuclear polymorphisms (SNPs) to predict risk for a disease or its behavior will be possible, but it will impose considerable technical and ethical problems (review, 7,8).

We will provide a short summary of recent advances in mRNA expression–based molecular disease classification and we will demonstrate the principle feasibility of gene expression–based disease categorization in the kidney. Finally, we will discuss strategies to translate this experimental approach to routine renal biopsy diagnostics.

Gene Expression Profiles Enable a Process-Oriented Disease Classification

Biomedical research is changing from an information-poor venture to an information-intensive undertaking. This is being
driven by the development of technologies that include DNA microarray analysis for measuring the transcriptional activity of cells, large-scale sequencing projects, and the development of tools that allow analysis of gene expression networks (9). The challenge to scientists and physicians seeking to understand the basis of disease is to adequately describe, integrate, and interpret the molecular events involved in the initiation and progression of the specific disease process. To address this, biology has begun to employ information processing and bioinformatics.

Researchers can now realistically expect to characterize a majority of the gene expression patterns associated with a given disease. Functional genomics aims to characterize discreet aspects of the regulatory patterns during genome-wide responses. The challenge of functional genomics in pathology is to turn expression and sequence data into information that can be used to help diagnose disease.

The central tenant of molecular diagnosis by expression profiling is that phenotypic differences between normal and diseased tissue are preceded and associated by changes in gene expression patterns. These patterns can be analyzed using cDNA microarray technology. Therefore, investigation of the expression profiles of diseased tissue should lead to novel phenotypes, thus affecting a diagnosis.

In this section, we will discuss some of the major developments in the area of expression profile–dependent disease classification. A general overview of the microarray technology has recently been provided in this Journal (10). A comprehensive glossary of genomic and bioinformatics terminology can be found on the Web at www.ornl.gov/TechResources/Human_Genome/glossary/glossary.html and www.library.csi.cuny.edu/~davis/Bioinfo_326/glossaries.htm

Bioinformatics Approach to Gene Expression Analysis

The use of molecular diagnosis of disease has been pioneered in oncology. Human tumors are diverse in their histopathologic phenotype, their clinical course, and their response to treatment. It is possible to explain aspects of their clinical features by characterizing the gene expression of tumors. By combing DNA microarray-based molecular profiling with data analysis/interpretation tools, a number of groups have successfully distinguished cancerous from normal tissue and detected subtypes within one cancer hitherto unknown by histopathologic classifications.

To separate the respective expression data into defined categories, "cluster analysis" was introduced into molecular biology. Cluster analysis is a term for numerical techniques that divide the objects of study (e.g., mRNA expression profiles) into discrete groups. These groups are based on characteristics of the objects. The clusters reflect biologic significance that can be related to the research questions being asked. For example, if a data set has a large number of relative gene expression values, as is seen in DNA array analysis, one of the first approaches is to group those genes that show similar expression patterns (11,12). Co-expressed genes are likely to have related functions (9). For this analysis, the algorithm employed, termed "similarity metric," must be able to adequately determine whether two expression profiles are similar to each other. The set of values that make up the expression profile for a single gene (extent of upregulation or downregulation, time course of expression, etc.) are defined as a series of coordinates that then define a vector. The similarity metric is essentially a measure that compares the length and/or direction in which the two individual expression vectors point.

Cluster Analysis can Group Gene Expression Parameters

Eisen et al. (12) described a clustering approach for the analysis of gene expression data. This is a bottom-up approach whereby single expression profiles are successively joined to form nodes (groups of related patterns), which in turn are then further joined to other nodes. The process is repeated until all individual profiles and nodes have been joined to form a single hierarchical tree. The advantage of this approach is that it is simple, the data are easily visualized, and coordinately regulated patterns can be readily determined by visual inspection. This technique has been used to group genes that were differentially regulated in human fibroblasts in response to serum stimulation. The clusters of genes identified were found to share common functionality (13).

Several Clustering Techniques Allow Gene Expression–Based Definition of Tumor Samples

Perou et al. (14) used the hierarchical clustering approach to paint a molecular portrait of human breast cancers. Variation in gene expression patterns in 65 surgical resection specimens of human breast tumors from 42 different individuals was determined using cDNA microarrays. A hierarchical clustering approach was used to group genes from the different samples on the basis of similarity in the pattern with which their expression varied over all samples. These analyses demonstrated great variation in the patterns of expression of the different tumor sample, with sets of genes showing independent patterns of variation. Furthermore, sets of coexpressed genes were identified according to which variation in the expression profile could be related to specific features of tumor biology. Using the intraexperimental variation of gene expression detected in paired samples from the same tumor as baseline, a set of 496 genes were identified that showed significant variation in expression between different tumors. This gene cluster could be used to identify subcategories of the tissue samples. The cancers could thus be classified into a number of different groups, including a basal epithelial group, an ERB-B2–overexpressing group, and a normal breast-like group. Further classification on the basis of estrogen receptor–positive and –negative tumors with characteristic gene expression profiles was also achieved.

Sorlie et al. (15) extended these studies by incorporating patient survival information. Using a technique known as self-organizing maps (SOM) (16), the authors searched for genes that correlated with patient survival. SOM computes a score for each gene that is a measure of its correlation with outcome. This score is a maximum-likelihood score. When the score is negative, higher expression correlates with longer survival; however, a positive score indicates that higher expression
correlates with shorter survival. By using this approach, a list of 264 cDNA clones was identified that showed significant correlation with patient survival. This set of clones was then analyzed using cluster analysis. Almost all of the cDNAs were found to fall into three categories: a basal epithelial cluster, a proliferation cluster, and a luminal cluster. These subtypes were then compared with overall survival and relapse-free survival to see if the different groups represent clinically distinct patient groups. These studies indicated a significant difference in survival between the subtypes, with the basal group having the shortest survival time. The patients with estrogen receptor–positive tumors were found to have the best prognosis. This ability to distinguish tumor subtypes by analyzing gene expression provides a basis for the diagnosis of breast cancer subtypes on the basis of biologic behavior. The combination of expression profiles and clinical information will hopefully permit accurate phenotyping of different tumor types, which may affect choice of treatment by highlighting resistance and sensitivity to specific treatments.

Golub et al. (17) have analyzed the expression of 6817 human genes in 27 acute lymphoblastic leukemias and 11 acute myeloid leukemias. They set out to use these data to build a class predictor, which, when challenged with expression data from an acute leukemia sample of unknown origin, would be able to accurately classify it into either of the previously seen classes. To do this, the authors first identified genes for which expression correlated to the specific type of leukemia and then tested whether the number of genes that appeared to have predictive value was greater than would be expected by chance. They identified roughly 1100 genes and conservatively chose the 50 with the highest scores to be used as part of a predictor analysis. Using these genes, they characterized an unknown sample with the following criteria: each gene was given a weighted vote for whether it would assign the sample to one class or the other. The vote was weighted as to how well expression of the gene could distinguish the two classes; in other words, a gene that can perfectly distinguish the two classes has a greater weight than one that distinguishes them less well. The weighted votes for all the genes were then summed to see to which class the predictor set of genes would assign the new sample. To test their technique, they took their 38 patient samples, built a “predictor” from 37 of them, and used the predictor to assign the missing sample. They repeated this for all 38 samples. Their technique was remarkably robust in this cross-validation approach, correctly assigning 36 of the 38 samples, with the other 2 having uncertain assignment. On an independent set of 34 leukemias, the technique made correct predictions for 29 of the samples.

**Gene Expression–Based Cluster Analysis Can Be Used as a Prognostic Tool**

Takashi et al. (18) used a cluster approach to profile renal clear cell carcinoma. The authors performed DNA microarray analysis on 29 renal tumors from patients with variable clinical outcomes. By employing cluster analysis, the investigations identified gene expression alterations that were common to most of the samples studied and some that were unique to clinical subsets. A significant difference was found in terms of gene expression between patients with a nonprogressive form of the disease versus patients with an aggressive form. A cohort of approximately 40 genes was found to accurately reflect this distinction. Used as a prognostic tool, the gene expression profiles were compared with traditional clinicopathologic staging. In 96% of the samples studied, the predicted prognosis was comparable to the observed clinical outcome, exceeding the reliability of staging. These data highlighted the existence of two clinically distinct forms of clear cell carcinoma, nonaggressive and aggressive form, which could be distinguished on the basis of gene expression profiles.

**Translation of Molecular-Diagnostic Strategies to Renal Disease**

Information obtained from gene expression studies in renal tissue should also lead to refinements of diagnoses, to reliable predictions of prognosis, and to improved selection of available therapeutic options. Before applying the molecular approaches described in the studies on tumor tissue, we should pause to consider the fundamental differences between neoplastic and renal disease processes.

In cancerous tissue, the gene expression profile is predominantly generated by clonal expansions of transformed cells, replacing the native cell population and potentially initiating an immune response within the affected organ.

In kidney disease, the gene expression profile is a consequence of transcriptional programs in intrinsic renal tissue comprised of diverse cellular types and, to a variable degree, by infiltrating inflammatory cells. Furthermore, the renal tissue is altered in a focal manner with coexistence of florid inflammatory processes, areas of tissue remodeling, and areas of fibrosis.

As compared with cancerous tissue, renal disease can be characterized by subtle changes in gene expression occurring within a microenvironment of the tissue that may become more apparent as the disease progresses. The changes in gene expression that characterize a pathophysiologic process may also be masked to a degree by non–disease-associated changes in gene expression that occur as a consequence of confounding factors (lifestyle, genetic background, age, etc.). These interindividual differences can be overcome by adequate training of the statistical algorithms used to assign diagnosis or classify the samples. By observing the gene expression profiles of sufficiently large groups of biopsy specimens, key differences that correlate most closely with disease should be identified.

**Specific Molecular Subgroups Can Be Obtained by Array-Based Gene Expression Profiling of Tubulointerstitial Damage in Human Kidneys**

As a proof of concept of what can be expected from gene expression analysis of renal tissue, we characterized a series of surgically removed human hydronephrotic kidneys with variable degrees of inflammation, fibrosis, and tubular atrophy and compared them with tissue from control kidneys. Gene expression levels were characterized by DNA macroarray, and sam-
amples were grouped by hierarchical clustering. A self-organizing map and average linkage hierarchical clustering, including automatically weighted voting, was used to analyze the data by applying the software programs CLUSTER and TREEVIEW (12). A renal pathologist unaware of the clustering data determined the degree of tubulointerstitial inflammation, fibrosis, and tubular atrophy using a semiquantitative scoring system (19).

The preliminary results suggest that average linkage hierarchical clustering can separate the gene expression pattern of the samples into three general groups (Figure 1): one group corresponds to the control tissue, and two subgroups were identified in the hydronephrotic kidney population. The cluster dendrogram separated the kidneys that showed pronounced chronic inflammation with low fibrosis scores from the kidneys showing high fibrosis-tubular atrophy and low chronic inflammation. A panel of 28 marker genes that could separate the samples was identified from the initial data set. Figure 1 shows the subset of these predictive cDNAs as a color-coded matrix after clustering.

These test results suggest that cDNA array techniques can be used to determine specific gene expression patterns within defined subsets of a renal disease. Further verifications of this marker set are clearly required within a population of renal biopsies with well-defined tubulointerstitial alterations and corresponding clinical information to assess their predictive strength.

Problems Associated with Molecular Diagnosis of Renal Biopsies

Compared with the studies using tumor material or nephrectomies, gene expression analysis in renal biopsies poses a formidable set of challenges.

I. Potential heterogeneity of classic renal disease entities
   a. Different diseases can show similar histopathologies. Renal diseases are classified according to descriptive histologic disease categories. This approach cannot exclude the possibility that more than one pathomechanism leads to an identical histologic picture, hiding different diseases under a comparable histopathology.
   b. Renal diseases produce multistaged lesions. Renal diseases are usually diagnosed at different stages of progression that may even coexist in the same biopsy.

II. Limited amount of tissue obtained by fine needle biopsy
   a. Renal biopsy is prone to sample bias. A renal biopsy represents a small fraction of the diseased organ, introducing a considerable sample bias. In addition, different nephron segments are contained in a biopsy in unknown proportions.
   b. Reproducible gene expression patterns must be obtained from the small amounts of tissue.

Heterogeneity of Renal Disease

Different Diseases Can Show Similar Histopathologies.

The definition of renal disease by morphologic criteria has developed into a detailed classification scheme. However, diagnosis based mainly on histology can limit the exact characterization of a disease, omitting functional implications, and often lacking sufficient accuracy to predict the response to therapy and its prognosis for the individual patient (20,21).

For example, the diagnostic group of focal and segmental glomerulosclerosis has been shown to consist of an idiopathic group as well as several genetic subgroups with distinct differences in the clinical picture, some of which are due to mutations in proteins associated with the slit diaphragm of podocytes (5,6).

Similarly, the most prevalent glomerular disease, IgA nephritis, is identified by a positive stain for IgA within the mesangium. The extent of mesangial hypercellularity and/or mesangial matrix expansion are not as yet major diagnostic factors. IgA nephritis with a pronounced increase in mesangial cells, matrix, and IgA deposition may be suspected to represent a distinct category within IgA nephritis (Figure 2, A through D).

Renal Diseases Produce Multistaged Lesions during Disease Progression. During disease progression, sequential histologic alterations have been correlated with clinical outcome. For example, the diagnosis of an interstitial nephritis is based on the presence of an inflammatory infiltrate (Figure 3, A and B) in the cortical and/or medullary interstitium. However, there are no universally accepted histopathologic methods to reliably judge the aggressive or bystander nature of an interstitial inflammatory infiltrate and its role in a defined disease state, e.g., interstitial inflammatory infiltrate in nephrosclerosis. In addition, different stages of infiltration and fibrosis frequently coexist in the same biopsy.

Glomerular damage and inflammation in lupus nephritis has been categorized into six classes. Spontaneous and therapy-associated transformations of morphologic types occur frequently. The pathogenetic processes that lead to these transformations of morphologic phenotypes are not known. Additional parameters of disease activity could add critical information for clinical decisions to the current classification of lupus glomerulonephritis.

The processes that lead to the development of renal disease can be broken down into a series of levels shown schematically in Figure 4. Even though different disease processes underlie the progression of the lesion, the end result can still be viewed as a common morphologic alteration.

As gene expression analysis and histopathology examine different levels of a disease process, expression profiling should help define renal disease categories and stages (Figure 4). Cluster algorithms have already been used to define clinically distinct subgroups using gene array profiles in cancer (see above).

To obtain and validate a set of marker genes that can define a renal diagnostic category, a comprehensive collection of tissue specimens and a databank containing corresponding clinical parameters, histopathology findings, and relevant follow-up data are required. This necessitates a considerable number of biopsies for each renal disease entity and requires a multicenter approach. The techniques required for the processing of biopsy samples for a comprehensive expression analysis in a multicenter set up have recently been established (see below and reference 22). A
Figure 1. Cluster dendrogram of hydronephrotic kidneys. Total RNA was isolated from kidneys immediately after surgical removal from nine hydronephrotic and four control kidneys (histologic verification of unaffected regions from tumor nephrectomies). Random primed radioactively labeled cDNA probes were hybridized to Panorama Human Cytokine Gene Arrays (Genosys; Sigma, St. Louis, MO) that were skewed toward genes representing molecules involved in cell-cell contact (integrins), matrix turnover (matrix metalloproteinases), and cytokines, chemokines, and their receptors. Hybridization signals were detected with phosphor image screens (Storm; Molecular Dynamics, Amersham Pharmacia, Piscataway, NJ), and expression levels were quantified using ArrayVision software (Imaging Research Inc., St. Catherine’s, Ontario). For normalization of the hybridization signals, the average intensity of all signals was used. Gene expression levels and samples were grouped by hierarchical clustering. A self-organizing map and average linkage hierarchical clustering, including automatically weighted voting, was used to analyze the data by applying CLUSTER and TREEVIEW. After processing the data, the dendrogram was visualized in TREEVIEW, where the tree structure reflects the relationship among the samples. In parallel to the molecular studies, a renal pathologist unaware of the clustering data determined the degree of tubulointerstitial inflammation, fibrosis, and tubular atrophy using light microscopy and semiquantitative immunohistochemical data. The dendrogram of the x-axis separates three groups of samples: (I) hydronephrotic group with low inflammation and high fibrosis score (fibrosis); (II) hydronephrotic group with high inflammation and low fibrosis (inflam); and (III) the control group (control).
The problem present to all studies on renal biopsy is the comparison of the diseased tissue to a normal population of kidneys. Most studies use the unaffected part of a tumor nephrectomy with the problem of histologically undetectable, tumor-induced alterations in this tissue. In addition, different processing of the surgically removed material compared with fine needle renal biopsies can result in gene expression alterations. Alternatives include baseline biopsies of kidneys immediately before transplantation, particularly from well-characterized living related donors. However, this tissue will also show gene expression alterations related to the methods of tissue preservation used before transplant. The occasional biopsy with normal histology and clinical data compatible with an extrarenal cause of the disease.

Figure 2. Different morphologic phenotypes of IgA nephritis suggest different subcategories of the disease. (A) Typical mesangioproliferative IgA glomerulonephritis with an increase in mesangial cell number and mesangial matrix (PAS). (B) The immunohistochemistry, done by alkaline-phosphatase, anti-alkaline-phosphatase method on paraffin-embedded formaldehyde-fixed tissue for IgA shows a strong granular mesangial label. (C) IgA nephritis with glomerulitis that shows only slight damage of mesangial areas (PAS). (D) The corresponding immunohistochemistry (same method as in panel B) depicts IgA deposits of varying sizes in the mesangial area.
symptoms can be considered normal. To obtain significant numbers of these rare biopsies, a multicenter approach is suggested.

**Figure 3.** (A) Interstitial infiltrates may be difficult to assess for their aggressive or bystander nature. Mononuclear interstitial infiltrate in a transplant biopsy without signs of florid vascular rejection and without infiltration of tubules by mononuclear cells is shown as an example. (B) Tubulointerstitial damage may be difficult to evaluate with regards to reversible or irreversible damage. Tubulointerstitial damage is shown in a biopsy with focal segmental necrotizing glomerulonephritis. The interstitial infiltrate is sparse to moderate; the tubules have collapsed with partly segment-specific differentiation, partly cuboidal cells. Some mononuclear cells infiltrate the tubular epithelia. Tubular basement membranes are wrinkled and slightly broadened due to tubular collapse.

**Figure 4.** Gene expression can detect multiple regulatory levels of renal disease development. Levels at which expression profiling may characterize a renal disease process are bold-faced. Arrow: leads to. Analysis of gene expression changes will describe a different level of disease development and can therefore supply adjunctive information to the histologic characterization of structural damage, shown in italics.

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**Limited Amount of Tissue is Obtained by Fine Needle Biopsy**

**Renal Biopsy Is Prone to Sample Bias.** Renal biopsies represent a minute fraction of the diseased organ. Considerable sample bias is inevitable. In glomerular disease, a representative number of glomeruli are required to establish adequate diagnosis. For example, in diagnosis of focal and segmental glomerulosclerosis diagnostic accuracy is dependent on the severity of the disease and the number of glomeruli in the biopsy section (23).

These sample biases will also affect the results of gene expression analysis. For the establishment of disease-specific molecular marker systems, sample bias can be overcome by analyzing a large population, resulting in a representative gene expression profile. However, this approach does not solve the diagnostic dilemma of the sample error in the specific biopsy case. Adjunctive techniques can address this problem, as shown by the implementation of electron microscopy to biopsy diagnostics of the nephrotic syndrome. This technique can detect specific alterations, e.g., characteristic podocyte changes, invisible by light microscopy. In parallel, the detection of molecules that already show differentially regulated gene expression in still histologically normal structures would be a useful diagnostic instrument (see Figure 4 and the Outlook section below).

**Reproducible Gene Expression Pattern Must Be Obtained from Minute Amounts of Tissue.** The small amount of tissue obtained by fine needle renal biopsies provides a challenge for subsequent characterization. Increasing the amount of renal biopsy tissue for molecular analysis is not feasible, given the low, but still significant risk of complications from this invasive procedure (24,25).

To overcome this problem, two potential strategies for molecular analysis of renal biopsies have emerged.

**Small Segments of Renal Biopsies Can Be Processed for Gene Expression Analysis.** With this approach, a segment of a renal biopsy is set apart specifically for gene expression analysis. To this end, an additional biopsy core has been obtained for mRNA expression (26,27). With the increase in sensitivity of mRNA analysis now available, as little as 10% of a biopsy core can be set aside for molecular analysis (see below and reference 22). Studies using lysates of the entire kidney biopsy may be confounded by tissue heterogeneity. More than 20 different cell types may be present in differing proportions in a given renal biopsy. Manual microdissection under a stereomicroscope is an effective approach that has allowed analysis of nephron segment-specific gene expression in animal models (28–33). In human biopsy studies, manual microdissection and PCR-based gene expression analysis of nephron segments has also been reported in several studies (26,34–38).

Another challenge in mRNA analysis is the inherent instability of the mRNA templates, particularly if material from clinical specimens that may not be processed immediately is used. A new class of RNase inhibitors, exemplified by RNA-later (Ambion), allows good preservation of RNA even when the samples are kept at room temperature for several hours.
This provides a convenient time window for processing the material. Using these agents, renal tissue and RNA will remain intact at \(-20^\circ C\) for more than 3 yr (unpublished observation). After such storage of the biopsy core, the nephron segments can still be manually microdissected and the tissue remains suitable for both immunohistochemical and gene expression analysis (22,39).

**Gene Expression Analysis Can Also Be Performed on Fixed and Processed Biopsies.** An alternative approach is to evaluate mRNA expression on material fixed and processed for routine diagnostic evaluation. Laser-assisted microdissection allows the selection of a defined histologic structure from the fixed biopsy slide, which can then be evaluated for gene expression. Thus histologic and molecular analysis can be performed in combination on routine histology preparations. Using the same nephron segments for histology and gene expression also allows a direct correlation of the information obtained. If frozen sections have been generated, RNA can be isolated using standard protocols (40–43). A technique for the isolation of RNA from formaldehyde-fixed, paraffin-embedded tissues has been described. This methodology enables mRNA analysis even after years of storage (43,44). As an added advantage, the mRNA expression profile can be correlated with the clinical course of the disease when archival material is used for retrospective studies.

This approach allows comprehensive archival sample banks to be recruited for specific questions on gene expression even in rare diseases. In addition, it will allow for the determination of the predictive strength of marker molecules identified in expression screening protocols on a large sample population. A major drawback of this technique is the low amount of mRNA harvested from a fixed tissue specimen and the time involved in the microdissection, limiting the number of genes available for analysis (43).

**Sensitive Assays for Renal Gene Expression Have Been Developed**

Two techniques are currently employed to quantify the minimal amount of RNA that can be obtained from fine needle biopsy.

**Gene Expression Can Be Analyzed in Renal Biopsies by RT-PCR.** PCR-based methods were employed on renal biopsy material shortly after the technology became widely available (34,45). These protocols require labor-intensive quantification procedure, *i.e.*, competitive RT-PCR (46), to obtain semiquantitative results. Despite these obstacles, gene expression profiles for specific cDNA have been generated and correlated with clinical parameters in a considerable number of studies.

For example, Peten and coworkers determined glomerular \(\alpha-2\) IV collagen mRNA expression in nephrectomies and could show a correlation with glomerulosclerosis (34). The authors could confirm these data on isolated glomeruli obtained from microdissected biopsies in a multicenter study (35). Several studies correlated gene expression levels in native biopsies with histologic parameters (27,36,37). Further correlations found included the Th1/Th2 chemokine expression profiles with the clinical outcomes in glomerulonephritis (47,48), the angiotensin-converting enzyme (ACE) expression with the ACE-genotype of the patients (49), and the renin mRNA levels with the medical therapy by ACE inhibition (50). In renal transplants, mRNA expression of inflammatory mediators has been shown to correlate with acute rejection (51–53). A comprehensive study examining the expression of 15 immune-activated genes was able to identify acute rejection with high sensitivity and specificity in a population of 60 renal allografts (54). Preliminary studies using protocol biopsies at set time points have started to examine mRNA expression after renal transplantation to detect subclinical rejection and the beginning of chronic graft dysfunction (55).

PCR-based methods have also been developed to allow the quantification of specific mRNA species by the determination of amplicon numbers after each PCR cycle (56,57). ‘Real-time’ RT-PCR gives an accurate determination of mRNA expression levels. This approach has been used to quantify mRNA species from single microdissected glomeruli (40,41,43) and even a single microdissected podocyte (58,59).

In summary, RT-PCR studies are applicable to the smallest renal biopsy samples, but they are still limited by the number of genes that can be examined.

**DNA Arrays Allow Comprehensive Expression Analysis of Renal Biopsies.** DNA arrays are the method of choice for comprehensive gene expression profiling. The major limitation is the requirement of several micrograms of high-quality RNA as starting material. Therefore, initial experiments did not use renal biopsies; instead, they generated RNA from kidneys of animal models (60,61) or nephrectomies (62). These studies described the co-regulation of functionally related genes by cluster analysis and could identify several unexpected pathways activated in glomerular and tubulointerstitial disease. One recent publication used entire renal biopsy cores to obtain array-based expression data in transplant rejection. A significant induction of ten mRNA species, including some cytokines, was detected (63). However, a detailed bioinformatic work-up of the expression data was not performed, and the expression differences were not verified by independent quantitative technique (*e.g.*, RT-PCR).

It appears that for DNA array-based nephron segment-specific analysis, the only option is to amplify the RNA before analysis. This approach, with amplification efficiencies of more than 1000-fold, has been used for laser microdissected tissue from frozen sections (64,65). A major concern is the linearity of the amplification across different mRNA species. With highly effective amplifications, even small differences in amplification efficiencies can result in significant distortions in the amount of cRNAs generated. Using a large scale screening approach, *i.e.*, of 10,000 cDNAs, an error rate of 5% will result in 500 false signals. Therefore, rigorous quality controls are mandatory. Modifications of the initial protocol have resulted in considerable improvements of the linearity of the assay (64).

Comparing the expression profiles generated before and after linear amplification, a high degree of reproducibility between identical samples analyzed in duplicate could be shown (correlation coefficient, 0.98) (66). This protocol enabled a com-
prehensive expression profiling in microdissected samples, identifying comparable populations of regulated mRNAs in amplified and unamplified prostate tissue. Confirmation of relevant mRNAs still should be performed by independent techniques, i.e., real-time RT-PCR (66).

The combination of linear amplification and DNA array may allow the generation of comprehensive expression data from defined nephron segments and uniform renal cell populations. Effective amplification protocols will also be a prerequisite for array-based diagnostic platforms in the future.

Outlook: The Integration of Molecular Tools into Renal Biopsy Diagnostics

Two potential strategies can be envisioned for the integration of gene expression profiling into the routine diagnosis of renal disease. Both protocols allow a nephron segment–specific gene expression analysis, and both would require an amplification step before quantification is performed.

Specific Molecular Markers Can Guide in Defined Diagnostic Problems

Renal biopsy processing and interpretation would follow the current routine procedures, including analysis of fixed, embedded material by light, immunologic, and electron microscopy. Molecular diagnostic information is obtained as an add-on procedure to aid in the differential diagnosis and subcategorization of disease (Figure 5A). Diagnostic marker molecules with a defined function in the disease process can be evaluated for differential mRNA regulation by real-time RT-PCR. The choice of these molecules has to be validated for sensitivity, specificity and predictive value for the specific diagnostic problem. As this protocol can use formalin fixed material, the markers can be analyzed in retrospective studies from kidney biopsy archives.

The differentiation between focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) may serve as an example. Conventional histology cannot make a distinction between these diseases if only nonsclerosed glomeruli are present in the biopsy. A differentially regulated mRNA in glomeruli from FSGS and MCD could separate these diagnoses. Transforming growth factor–β and α-actinin 4 have been reported as potential markers for the differential diagnosis of FSGS versus MCD (67,68).

According to this principle, one can envision a panel of 2 to 5 markers to be established for each diagnostic problem. This approach is flexible and can be complementary to conventional diagnosis at many levels. It can be added to the biopsy analysis, does not require special tissue processing, and uses consecutive sections of the same material evaluated by histopathology. If mRNA expression analysis is not available locally, the tissue could be sent to a reference center for molecular diagnostics. Molecules identified by these approaches could also be incorporated into the routine immunofluorescence evaluation of the biopsies if protein expression follows mRNA regulation.

Molecular Marker Profiling and Routine Biopsy Work-Up as Parallel Strategies

With this approach, conventional diagnosis and gene expression profiling would be performed in parallel. The independently obtained diagnostic predictions would have to be combined to obtain an integrated diagnosis (Figure 5B).

As high-quality RNA is required for array-based analysis, the appropriate harvesting of renal biopsy tissue for microdissection and gene expression profiling would be necessary (see above and reference 22).

This could be achieved by obtaining two biopsy cores, with at least one core being used for evaluation by routine histologic diagnostic procedures, establishing the conventional diagnosis, and the remainder being used for expression analysis. Molecular analysis would be performed after nephron segment dissection and linear amplification of the RNA on a DNA array containing a set of molecular diagnostic markers. This marker set would have to be generated initially by large-scale
expression profiling on a comprehensive tissue bank as suggested in the previous section.

Initially, expression profiles will most likely be generated for the most abundant diseases (i.e., IgA nephropathy) and may allow the definition of clinical subgroups with different disease courses. As more expression data from different diseases are generated, molecular markers may be able to identify precise disease fingerprints and potential mechanisms underlying the pathology of specific diseases. These markers can be combined on a small-scale expression profiling platform, most likely a “renal diagnostic chip,” evaluating a few hundred RNAs or less.

For the growth of these techniques, an exchange platform for gene expression profiles, as currently under development by the Microarray Gene Expression Data Group (MGED Group; www.mged.org/), would greatly enhance the development of predictive renal disease marker sets by combining the limited resources available.

Conclusions

With application of gene expression profiling to mainstream kidney research, a rapid growth of data for the characterization of renal diseases is anticipated. To use this information for diagnostic applications, the extraction of clinically useful molecular marker sets will be the initial challenge. For their validation, the study of large representative populations of specific renal disease samples will be imperative. The overall goal is to obtain complementary information on renal biopsies that allow a more stringent prediction concerning diagnosis, prognosis, and differential therapy.

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