Genomics of chronic allograft injury

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Chronic allograft injury (CAI) is common after kidney transplantation in which immunological (e.g., acute and chronic cellular and antibody-mediated rejection) and nonimmunological factors (e.g., donor-related factors, ischemia–reperfusion injury, polyoma virus, hypertension, and calcineurin inhibitor nephrotoxicity) have a role. Despite the new Banff pathological classification, histopathological diagnosis is still far from being the 'gold standard' to understand the exact mechanisms in the development of CAI, which may lead to appropriate treatment. Microarray is a powerful technology that detects thousands of genes simultaneously and might be an important tool in elucidating patterns for mechanism, diagnosis, prognosis, and treatment of complex, multifactorial diseases, such as CAI. In this review, we discuss the studies that applied microarray technology in kidney transplant patients with CAI.

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Chronic allograft injury (CAI) is common after kidney transplantation and has been documented in more than 50% of the recipient's 1-year protocol biopsies and is the leading cause of graft loss after the patient's death. CAI is a multifactorial process in which immunological (for example, acute and chronic cellular and antibody-mediated rejection) and non-immunological factors (for example, donor-related factors, ischemia–reperfusion injury, polyoma virus, hypertension, and calcineurin inhibitor nephrotoxicity) have a role.[1] However, these factors are not mutually exclusive and CAI most likely results from the combination of multiple factors, and it is often difficult to determine the contribution of each factor. Previous terminology, namely, chronic allograft nephropathy (CAN), was eliminated in the 8th Banff Conference in 2005 because of the misconception that 'CAN' is a specific disease rather than just another term for nonspecific scarring of all causes of chronic allograft dysfunction with fibrosis, which inhibits the physicians to determine accurate diagnosis and treatment.[2] The new classification replaced the term CAN with interstitial fibrosis and tubular atrophy (IF/TA), no evidence of any specific etiology, and mandates recognition and notation of specific morphological features, such as polyomavirus nephropathy (immunostaining for SV40 antigen), calcineurin inhibitor toxicity (arteriolar hyalinosis with peripheral hyaline nodules in the absence of hypertension or diabetes), and recurrent or *de novo* glomerular disease, and added the term 'chronic active antibody-mediated rejection' to biopsy samples showing transplant glomerulopathy (TGP), peritubular capillary basement membrane multilayering with positive C4d staining and circulating donor-specific anti-HLA antibodies, and 'chronic active T-cell-mediated rejection' (chronic allograft arteriopathy with mononuclear cell infiltration in arterial intimal fibrosis and formation of neo-intima). However, this new classification is still far from being the 'gold standard' to understand the exact mechanisms in the development of CAI, which may lead to appropriate treatment. TGP may develop in the absence of donor-specific anti-HLA antibodies and C4d staining,[3] and previous study showed glomerular infiltraton by CXCR3 + ICOS + -activated T cells in TGP biopsies, suggesting an active T-cell response to glomerular antigens.[4] Calcinuerin inhibitor toxicity has been shown to lead to arteriolar hyalinosis and subsequent glomerulosclerosis in selected cohort studies.[5] However, it is difficult to attribute arteriolar hyalinosis and hyaline nodules to

calcineurin inhibitor toxicity in the usual context of renal transplantation, in which 80–90% of transplant recipients are hypertensive and 50–60% have pretransplant or posttransplant diabetes mellitus. The current Banff classification forbid assessment of interstitial inflammation in areas of IF/TA and no therapeutic intervention has been taken. However, Cosio *et al.*[6] demonstrated lower allograft survival in patients who had IF/TA and inflammation compared with patients who had only IF/TA without any significant infiltrates. In addition, interpretation of biopsies is too subjective and the reproducibility of the lesion scoring and pathological diagnosis is <50–70% between pathologists. These findings indicate the necessity of diagnostic studies at molecular level, on top of histopathological examination of the biopsies.

GENOMICS

Traditional genomics methods, such as polymerase chain reaction, limited the investigators in the study of certain candidate genes; these studies were more hypothesis-driven experiments. Recent advances in genomics technology using microarrays have made it possible to detect expression patterns of thousands of genes simultaneously and shifted experiments toward large-scale hypothesis-generating data collection. Two types of microarrays, using high-density array of complementary DNA or oligonucleotide probes (Affymetrix GeneChip; Santa Clara, CA), have been successfully used to analyze the gene expression patterns of different types of cancers, autoimmune and inflammatory diseases. Although the diseased and control RNA samples are cohybridized into one array after being fluorescently labeled by two different colors in the complementary DNA microarray system, samples are hybridized into separate arrays in a single-color assay in the oligonucleotide array system. After the samples are scanned, an image analysis software determines the pixel intensity of the spots, and hybridization intensities for each DNA sequence. A large set of data representing a genome-wide gene expression profile is generated, which is also called 'transcriptome'. Following data normalization and data filtering to eliminate genes expressed below a certain threshold, the differences in gene expression between experimental and control samples are identified as upregulated and downregulated with a fold change. There are different data analysis methods to investigate gene expression patterns of the samples. Statistical analysis of microarrays and unsupervised hierarchical clustering are the most common methods used to group samples on the basis of their expression profile.

MICROARRAY STUDIES IN HUMAN KIDNEY TRANSPLANTATION

The application of microarray technology in human kidney transplantation is recent. The first study was published in 2001,[7] which reported a set of genes that are persistently upregulated in kidney biopsy samples from patients with acute rejection compared with normal kidney transplant biopsy specimens, using oligonucleotide arrays. Sarwal *et al.*[8] demonstrated the molecular heterogeneity of acute rejection, with at least three possible distinct subtypes indistinguishable by light microscopy, using complementary DNA microarrays. Dr Halloran's group developed pathogenesis-based transcript sets for cytotoxic T lymphocytes, B lymphocytes, natural killer cells, macrophages, endothelium, and interferon-γ-inducible transcripts to examine the gene expression profiles of kidney transplant biopsies by microarrays and published a series of experiments.[9] They studied 186 consecutive clinically indicated biopsies and built a classifier to distinguish rejection from non-rejection using predictive analysis of microarrays. Comparing predictive analysis of microarrays diagnoses with histopathological diagnosis by Banff classification, there was a 20% disagreement that the authors suggested the necessity of a new system combining molecular and histopathological findings.[10]

Microarray studies have been applied not only to investigate the gene expression patterns of transplant kidney biopsies but also peripheral blood cells of patients with rejection.[11,12] 'Operational tolerance', a clinical state defined as stable graft function in the absence of any immunosuppressive medication for more than 1 year, is rare in kidney transplant recipients. Brouard *et al.*[13] used microarray analysis of the peripheral blood cells of 17 patients in this state and identified a 'tolerant footprint' of 49 genes.

A recent review article by Ying and Sarwal[14] discussed the studies that applied microarray technology to transplantation in detail.

MICROARRAY ANALYSIS OF KIDNEY TRANSPLANT BIOPSIES WITH CAI

The first study of microarray analysis of CAI was published by Scherer *et al.*[15] in 2003. The authors investigated the gene expression profiles of 17 protocol kidney biopsy samples with normal histopathology at 6 months after transplantation by oligonucleotide microarrays in patients who were enrolled in a multicenter trial involving Certican (everolimus). Nine patients (all in the everolimus group) developed chronic rejection at 12 months after transplantation, and a set of 10 genes (eight upregulated and two downregulated) from 6-month protocol biopsy samples with normal histopathology were considered to predict the development of chronic rejection. Those genes were APRIL (acidic protein rich in leucins), OBCML (opiate-binding protein–cell adhesion molecule-like), the tumor suppressor gene *NPRL2*, cytokeratin 15, homeobox gene B7, prolactin receptor, and guanine nucleotide-binding protein γ7. The same group compared CAN biopsy samples with normal renal allografts and demonstrated different gene expression profiles.[16] Donauer *et al.*[17] demonstrated two distinct subsets of chronically rejected transplants in 13 transplant nephrectomy samples by complementary DNA microarrays. However, transplant nephrectomies were performed electively after patients initiated dialysis and their immunosuppressive medications

were minimized so that probably most biopsies had findings of acute rejection on top of chronic rejection. Flechner *et al.*[11] showed significant upregulation of genes responsible for immune/inflammation and fibrosis/tissue remodeling in protocol biopsy samples of patients with CAN in a randomized prospective trial comparing cyclosporin and sirolimus treatment at 2 years after transplantation by oligonucleotide microarrays.

We have investigated gene expression profiles of 16 kidney transplant biopsy samples with CAN by high-density oligonucleotide microarrays, and compared them with six normal transplant biopsy specimens.[18] Eight CAN biopsy samples showed nodular arteriolar hyalinization and one was positive for C4d staining. Hierarchical clustering analysis of the 22 biopsies revealed differential gene expression patterns in CAN compared with control biopsies. However, microarray analysis did not reveal differential gene expression patterns in patients with or without arteriolar hyalinization. There was differential expression of profibrotic and growth factors; although transforming growth factor-β-induced factor, thrombospondin 1, and platelet-derived growth factor C were upregulated, vascular endothelial growth factor, epidermal growth factor, and fibroblast growth factors 1 and 9 were downregulated. Immunopathological examination of biopsies revealed strong transforming growth factor-β but decreased glomerular vascular endothelial growth factor expression in CAN. Mas *et al.*[19] conducted a similar study to compare the gene expression patterns of 11 CAN biopsy samples with seven normal kidneys and reported that genes related to fibrosis, extracellular matrix deposition, and immune response were upregulated in CAN biopsy specimens. The authors further reported the gene expression profiles of 17 biopsy samples with IF/TA compared with six normal allografts and 24 normal kidney biopsy specimens[20] and also conducted a non-parametric meta-analysis approach for combining the microarray data of their studies with ours.[21]

Park *et al.*[22] studied baseline and 1-year protocol biopsies of 15 living-donor kidney transplant recipients, in which seven were histologically normal and eight had subclinical IF/TA at 1 year. All 1-year biopsy specimens showed differential expression of 3578 probe sets compared with baseline specimens, and most upregulated genes were linked with inflammation, immunity, and response to injury. Despite normal histology, there was ongoing injury response and inflammation microarray findings in 1-year biopsies, and gene expression patterns of those biopsy samples were closer to those of 1-year biopsy samples with IF/TA compared with baseline biopsies.

Dr Halloran's group investigated 177 renal allograft biopsies for expression of B-cell-associated transcripts and immunoglobulin transcripts and demonstrated a correlation between interstitial inflammation and IF/TA in biopsies performed more than 5 months after transplantation.[23] Sis *et al.*[24] studied 119 endothelial-associated transcripts by microarrays in 173 clinically indicated biopsy samples. Interestingly, only 40% of kidneys with high endothelial-associated transcripts expression and chronic active antibody-mediated rejection had C4d positivity. This study suggested that chronic active antibody-mediated rejection can be diagnosed with demonstration of circulating alloantibody and high endothelial-associated transcripts expression by microarrays without C4d positivity, and predicts poor allograft outcome.

Ashton-Chess[25] used the microarray data of four previously published microarray studies[11,15,17,18] to identify common biomarkers of late graft injury demonstrating a good example that microarray experiments are hypothesis generating. The authors showed increased Tribbles Homolog (TRIB1) mRNA, immunoproteasome beta subunit 10 (PSMB10), and toll-like receptor 4 in the blood and allograft of patients with chronic active antibody-mediated rejection. mRNA expression of TRIB1 in 76 allograft biopsy samples and 71 blood samples revealed increased expression in TGP biopsies with positive C4d and donor-specific anti-HLA antibodies but not in C4d-negative and donor-specific anti-HLA antibody-negative TGP biopsies, as well as in biopsies showing calcineurin inhibitor toxicity, IF/TA, and normal histology. Similar differential TRIB1 mRNA profiles were observed in peripheral blood cells of renal transplant recipients but not in their urine samples.

INFORMATION OBTAINED FROM MICROARRAY ANALYSIS OF SEQUENTIAL PROTOCOL BIOPSIES

Most published studies using microarrays in the context of CAI have been cross-sectional in nature or were performed on biopsy samples taken for either acute or chronic graft dysfunction.[18,19] Although some studies have used protocol biopsies, the data from the early transplant period (that is, <12 months after transplantation) have been limited.[11,22] The ability to identify a gene expression profile in the early transplant period before significant histological damage has occurred is an attractive concept, as there is the potential for altering the disease process, which is difficult to carry out once significant fibrosis has occurred. Preliminary studies suggest that an early predictive gene pattern may be possible to identify. In a pilot study, 18 patients underwent protocol renal transplant biopsies at 0, 1, 3, and 12 months after transplantation. When biopsies were grouped according to the presence or absence of fibrosis at each time point, there was differential gene expression between those biopsies with IF/TA and those with 'normal' histological appearance. The differentially expressed genes could be divided into three broad groups: fibrogenic genes, immune-related genes, and genes involved in cell function and metabolism, such as heat-shock protein, growth factors, and G-protein signaling molecules.[26] Fibrogenic genes included genes involved in collagen production, fibroblast activation, extracellular matrix production, and remodeling. Immune-related genes identified included complement, natural killer cell, and T-cell-related genes.[26] This proof-of-concept study demonstrated that genetic profiles predictive of progressive fibrosis

could be identified at early time points when graft histology was unremarkable. Immune-mediated factors were present in the early transplant period even if graft inflammation is not readily identified by histology. This may be proven to be a predictor of subsequent graft fibrosis, as histological evaluation of protocol biopsies have identified early alloimmune-mediated graft inflammation and subclinical rejection as major causes of graft fibrosis.[27]

Gene array studies have the potential to shed light on the underlying pathogenesis of fibrosis in following renal allograft injury. Histological and clinical studies have shown that the pathogenesis is multifactorial, especially as fibrosis is the end process of many forms of injury. Some of these factors are likely to be donor related and genome-wide analyses of donor implantation biopsies have identified several putative predictive gene patterns associated with ischemia–reperfusion injury.[28–30] These include markers of inflammation, as well as antiangiogenic factors, although no definitive predictive pattern of ischemia–reperfusion injury or profibrotic factors has been demonstrated definitively.[31] In other circumstances, fibrosis is the end process of tubular cell injury. Recently, specific pathways of fibrosis such as epithelial-to-mesenchymal transition (EMT) have been postulated as a major cause of fibrosis in renal allografts. EMT is a highly regulated process whereby epithelial cells go through a series of programmed phenotypic changes including loss of epithelial markers and function, migration into the interstitial space, and morphosis into myofibroblasts. Studies suggesting that EMT was an important mechanism of graft fibrosis were generally cross-sectional in nature, relied heavily on for-cause biopsies relatively late in the history of the disease, and used immunohistochemical evaluation of epithelial and mesenchymal markers to define EMT.[32,33] In a study of 24 patients who had undergone protocol biopsies at 1, 3, and 12 months, a series of complementary technologies including genome-wide microarray analysis were used to investigate the role of EMT as a cause of fibrosis early after transplantation. Although immunohistochemical evidence of loss of some epithelial markers and the appearance of mesenchymal markers, such as α-smooth muscle actin and S100, was apparent in some tubules, interrogation of gene arrays for 173 EMT-associated genes did not detect a profile consistent with EMT being a major biological process early after transplantation.[34] IF/TA was not associated with immunohistochemical or gene evidence of EMT, rather it was associated with subclinical immune activity. As well as not supporting EMT as an important driver of graft fibrosis, genome-wide analysis allowed other hypotheses to be investigated at the same time. For instance, it identified immune-related activity as a potentially greater inducer of fibrosis than EMT. As with all the studies mentioned in this review, complementary technologies such as immunohistochemistry and quantitative PCR are essential to the design of any gene array study in order to confirm, or question, the conclusions drawn from the genome-wide analysis.

LIMITATIONS OF MICROARRAY STUDIES IN TRANSPLANTATION

Microarray technology has been applied in CAI patients in a limited number of studies involving a limited number of patients. This is one of the main reasons for weak overlap between gene lists from different studies. It would be very difficult to evaluate vast quantities of data to reach a meaningful conclusion in a limited number of heterogeneous groups of patients receiving different immunosuppressive medications to understand a very heterogeneous disease such as CAI. The other discrepancy in the outcomes of the studies using human kidney samples is the tissue sampling differences, as biopsy samples contain a mixture of different cell and tissue types, involving varying proportions of muscle, capsule, cortex, and medulla of the kidney. This problem can be overcome by using laser-capture micro-dissected tissue subtypes, although it would be technically difficult to obtain an adequate amount of RNA in a small biopsy sample.

The data analysis of microarrays is still in its infancy and there is no universally accepted method. The studies discussed above used different microarrays, experimental settings, data analysis, thresholds for data filtering, and statistical analysis. Another question is whether there is too much data in microarrays analyses? In an array of 10,000 elements, even at 95% confidence ($P<0.05$), 500 significant genes may be found purely by chance. Gene expression levels may also not correlate with protein levels and posttranslational modifications cannot be measured by microarrays. Another concern is that some genes considered undetectable by microarray can be detected by real-time PCR. Park and Stegall[35] studied 81 human kidney biopsy samples and showed that a majority of cytokine-related genes are not detectable by microarray but by real-time PCR. Allanach *et al.*[36] compared 13 potentially diagnostic genes by microarray and real-time PCR in renal allografts and showed that 10 out of 13 correlated well. Exceptions included fas ligand and CD8B1 microarray probe sets.

CONCLUSION

In summary, microarray is a powerful technology that detects thousands of genes simultaneously and might be an important tool in elucidating patterns for mechanism, diagnosis, prognosis, and treatment of complex, multifactorial diseases, such as CAI. However, for microarray studies to define certain subgroups of patients for diagnosis, prognosis, and management of CAI, or to find candidate genes, novel pathways, and/or surrogate markers, hundreds of biopsies should be performed in multicenter collaborative studies in a prospective manner, starting with microarray analysis of transplant kidney biopsies before implantation and follow-up protocol biopsies until CAI develops.

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