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REVIEW

Microarrays: a monitoring tool for transplant patients?

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Summary

Microarray technology holds a distinct advantage over traditional genomic methods, with the unique capability to rapidly generate multiple global gene expression profiles in parallel. This technology is quickly gaining widespread use in many areas of science and medicine because it can be easily adapted to study many experimental questions, particularly relating to disease heterogeneity. Microarray experiments have begun to advance our understanding of the underlying molecular processes in solid organ transplantation; however, several obstacles must be overcome before this technology is ready for application in the clinical setting. This article will review the current applications of microarray technology in the field of transplantation, and discuss the potential impact of this technology on monitoring of solid organ transplant recipients.

Introduction

The development of microarray technology has been a revolutionary advance in the field of genomic research, advancing our ability to characterize human disease and pathologic states. Since the completion of the Human Genome Project, the focus of genomic research has shifted toward functional genomics. Microarrays are powerful tools, which generate transcriptional profiles by simultaneously analyzing the expression of tens of thousands of genes. This technique of genome-wide scanning identifies changes in gene expression which occur in different pathologic states, providing a tool to gain insight into the underlying biological pathways in both health and disease. Previously, genomic research was restricted to traditional methods such as polymerase chain reaction and probe-based assays, which limited investigators to the study of individual candidate genes in isolation. High-throughput methods such as microarrays have expanded our ability to study complex disease processes, allowing genes to be studied in the context of other genes, providing insight into the underlying molecular pathways. Although oncology is by far the field with the most data generated by transcriptional studies [1–6], many other fields have begun to follow their lead, including studies in cardiovascular disease [7,8], Alzheimer's disease [9,10], rheumatologic disorders [11,12], and more recently, glomerular kidney disease [13,14] and organ transplantation. The major focus of this article is the current application of DNA microarray technology to the field of solid organ transplantation.

Microarray construction and technique basics

A microarray is a high-density array of complementary DNA (cDNA) or oligonucleotide probes immobilized on a solid support. The original method of 'genechip' construction consists of gridding cDNA fragments, typically approximately 500 bp in length, onto glass slides, which can accommodate as many as 50 000 genes on a single slide. Using a dual-color fluorescent dye system, relative gene expression in a sample is determined by comparing hybridization of cDNA probe sequences and reference samples. Currently, the most commonly used Affymetrix GeneChip® system (http://www.affymetrix.com/index.affx) photolithographically synthesizes millions of oligonucleotide probes on a coated quartz surface. This platform utilizes a single-color system to detect expression level differences. The short probe length, approximately 20–25 bp, is designed for higher specificity for DNA targets by increasing the ability to distinguish between near-identical sequences, thus decreasing the problematic

cross-hybridization of related genes more frequently encountered with cDNA microarrays. Reproducibility is enhanced by the use of multiple oligoprobes for each expression measurement. However, because of the short length and stringency of the probe sequence, this platform, may, in fact, not work as well for identification of closely related gene family members and polymorphic genes, such as genes in the HLA family. Recently, longer oligonucleotide probes, typically ranging between approximately 45–90 nucleotides in length, have been introduced (http://www.agilent.com/about/index.html, https://products.appliedbiosystems.com/) which reportedly maintain the higher specificity of oligonucleotide probes while allowing for greater identification of super gene families. Oligoarrays by Applied Biosystems utilize a novel chemiluminescent detection system and are composed of 60 mer geneprobes derived from both the Human Genome Project public domain database as well as the Celera Genomics dataset, which includes 8000 additional genes. Other formulations of microarrays are becoming more widely applied to solid organ transplantation research including single nucleotide polymorphism arrays (http://www.affymetrix.com/products/application/dna_analysis_products.affx), exon arrays (http://www.exonhit.com/html/company/index.htm), and transcriptome-specific arrays. Exon-level expression profiling should enhance biomarker discovery efforts by addressing the complexity of alternative splicing in gene transcription, which is problematic with whole-genome arrays. Arrays are manufactured by robotic technology at several commercial companies, and can also be generated locally at academic institutions (http://cmgm.stanford.edu/pbrown/mguide/index.html). Currently, several companies are also developing customized multiplex systems that contain a smaller number of selected and highly informative genes (http://www.agilent.com/about/index.html, http://www.genexpbiosciences.com/index.html). These platforms allow for the simultaneous processing of large numbers of patient samples, further advancing this technology toward large-scale application.

New lessons learned from microarrays

The progression toward high-throughput data production and analysis represents a global paradigm shift from *hypothesis-driven* experiments toward large-scale *hypothesis-generating* data collection. As a result, our understanding of biologic pathways is enhanced by the ability to identify nonbiased *expression patterns* of thousands of genes, including those with down-regulated as well as up-regulated transcription during pathologic states, rather just studying quantitative responses of single genes or groups of genes. Transplantation is a complex immunologic state involving constant interaction between the graft and the recipient's immune system. Numerous interrelated immune pathways are responsible for the delicate balance between graft rejection and acceptance, and are further complicated by manipulation by immunosuppressive medications. These precise molecular mechanisms remain poorly understood, specifically the *interplay between co-regulated immune pathways and gene families* that may as yet be 'unlinked', based on single gene analyses. However, the evolution of the field of functional genomics, including microarray technology, has begun to uncover these intricate gene regulation events, and thus offer new insights in to the pathogenesis of post-transplant clinical events.

Clinical variability in disease presentation and behavior is universally observed in the practice of medicine; however, few tools exist to allow clinicians to stratify patients by these criteria. Greater understanding of the underlying *molecular heterogeneity* of pathologic states which occur following transplantation will allow us to develop methods to obtain critical information regarding disease progression, and treatment response, and clinical outcome. Genomic profiling provides the link between biological mechanism and clinical phenotype by elucidating the underlying molecular processes responsible for the variability in clinical behavior and outcome, such as acute rejection (AR), chronic rejection (CR), drug toxicity (DT), ischemia-reperfusion injury, delayed graft function, infection, and tolerance (Fig. 1).

Controlled studies in transplantation only exist by the creation of *animal models*, which provide invaluable information regarding the immunologic injury and regulatory pathways. Animal models can be manipulated in a controlled environment to achieve a particular desired clinical phenotype with ideal controls (e.g. isografts to control for effect of ischemic injury), and are usually not limited by access to tissue samples or sampling frequency. Species-specific gene-chips are commercially available for use in such studies, and occasionally, cross-hybridization of higher species may also be accomplished on human arrays (Table 1). Mechanistic information obtained from these studies has potential for application toward hypothesis-driven studies in humans, as well as generating studies of therapeutic strategies to improve clinical outcomes. For example, data from a rat model by Schuurs *et al.* [22] suggest that alterations in gene expression, particularly in inflammatory and coagulation pathways, occur in response to brain death and may contribute to the higher rate of primary nonfunction in deceased donor versus living donor renal transplant. Further study of these mechanisms in humans can lead to targeted interventions aimed at increasing organ viability. Other examples include 'knock-out' mouse models for known critical

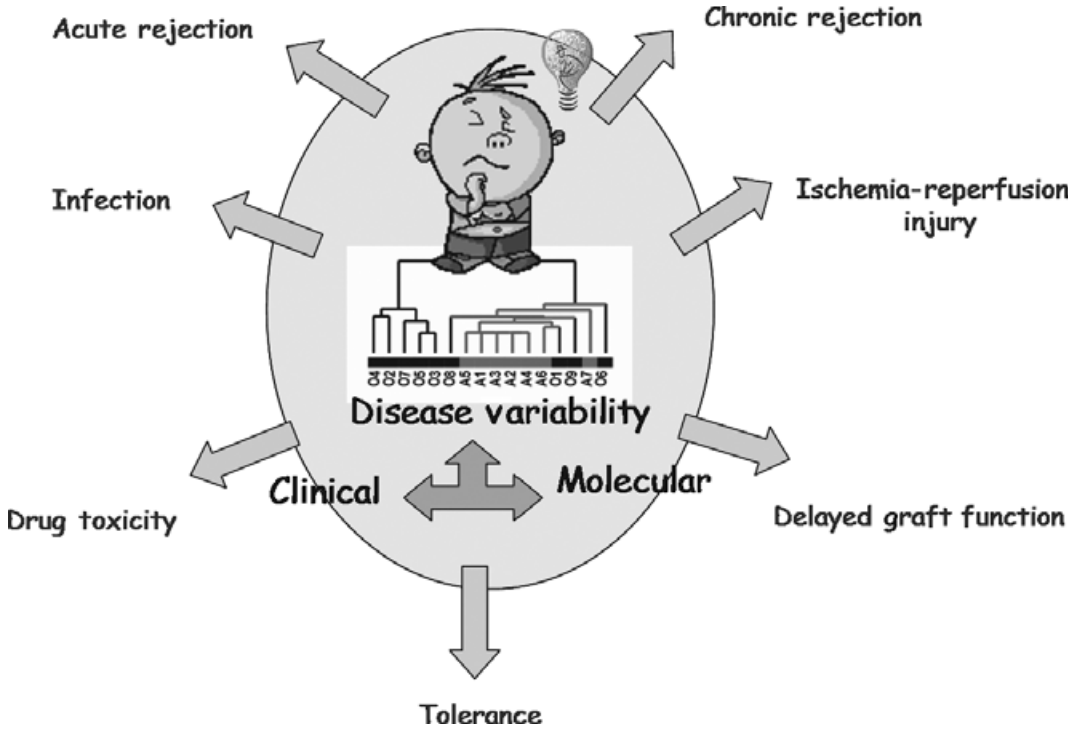

Figure 1 Complexity of microarray analysis in solid organ transplantation.

pathways in the alloimmune response, specifically interferon-gamma [31] and chemokine receptor-5 (CCR5) [25]. By uncovering alternate pathways involved in AR, researchers can characterize mechanisms which are typically buried in the redundancies of the immune response in order to identify novel therapeutic targets in the immune system.

The recent literature in *human* microarray based studies in solid organ transplantation is summarized in Table 2. Several independent groups have demonstrated distinct gene expression profiles, which correlate with different phenotypes in transplantation. The majority of these studies focus on AR, from which common molecular processes are consistently identified across different organs (kidney, heart, lung, and liver), such as up-regulation of immune-mediated pathways (HLA and cytokine genes), inflammation, apoptosis, cell-cycle regulation, and transcription regulation [39,40,43,48]. On the other hand, little overlap exists for the actual significant genes themselves in the individual studies, which may be a result of different platforms used in different studies, different density and composition of gene probes on the platforms, different sample handling and processing protocols, and most importantly, lack of adequate sample size in many studies for adequate biostatistical analysis. Key studies in solid organ transplantation are discussed below.

Acute rejection

It is well recognized that an AR is a heterogeneous process with variable clinical outcome and treatment response. Microarray analyses have identified unique alterations in gene expression profiles, which occur during AR episodes. Changes in the expression of genes responsible for a variety of cell functions, including immune activation, cellular proliferation, cell cycling, and apoptosis, were identified in kidney biopsy tissue [39,45], bronchoalveolar lavage (BAL) samples from lung transplants [48], and peripheral blood from cardiac transplants [40] with AR. Transcriptional microarray profiling has uncovered *molecular* heterogeneity in renal transplant rejection, identifying distinct molecular signatures, which are not distinguishable by light microscopy, and which correlate strongly with treatment response and risk of future graft loss [43]. Profiles of patients with the highest risk of graft loss include increased expression of genes implicated in infiltration and activation of lymphocytes, apoptosis, transcripts from a variety of inflammatory cells including cytotoxic T cells, natural killer cells, and macrophages as well as a dominant signature for B cells (CD20, CD74, immunoglobulin heavy and light chains, and other molecules associated with B-cell receptors). In the absence of a strong correlation with biopsy C4d staining, these latter

Table 1. Relevant animal studies in solid organ transplantation, descriptive for array platform, tissue source, experimental design, and key findings.

Author	Journal	Organ, species	Platform	Tissue	Phenotype	Experiment and website for raw data	Results/genes and pathways
Kitagawa-Sakakida *et al.* [15]	J Heart Lung Transplant 2005	Heart, rat	Oligonucleotide	Graft	Chronic rejection (CR)	Allogeneic cardiac transplantation (WKY to LEW rats); retransplantation into donor or T-cell deficient rats on day 3 or 5	B-cell genes, mast cell genes, and lipid metabolism
Aavik *et al.* [16]	FASEB 2005	Carotid, baboon	Human complementary DNA (cDNA)*	Carotid artery	Nonimmune denudation injury	Overstretch injury and endothelial denudation of baboon carotid artery (http://microarray-pubs.stanford.edu/DN_Treg/)	Apoptosis, vasoconstriction (early), cell migration/ proliferation, inflammation, energetics, extracellular matrix, and vasodilatation (late)
Lee *et al.* [17]	J Immunol 2005	Heart, mouse	cDNA	Graft	Tolerance	Mice preinfused with regulatory cells or mutant clone before heterotopic heart transplant with major histocompatibility complex (MHC) mismatched strain	Cell proliferation/survival, immune regulation, chemotaxis; downregulated genes of antigen presentation, apoptosis, and signal transduction
Yamane *et al.* [18]	AJT 2005	Lung, rat	Oligonucleotide	Graft	Ischemia/reperfusion	Lewis rat lung grafts – stored for 6 or 24 h, transplanted, reperfused for 2 h	Upregulated transcription factors, adhesion molecules, pro-coagulant factors and pro-inflammatory cytokines
Liu *et al.* [19]	Transplant Proc 2005	Heart, rat	Oligonucleotide	Graft	Tolerance	Allochimeric MHC Class I molecule delivery versus control	Downregulation of IL-4, IL-6, IFN-g, TNF, TGF-b binding; up-regulation of granzyme, IL-10, IL-15, CD4, cell activation, immune response, T-cell activation
Lande *et al.* [20]	AJT 2005	Lung, mouse	Oligonucleotide	Graft	AR/obliterative airway disease (OAD)	Mouse model of OAD-MHC mismatched and MHC identical controls; grafts removed days 4, 14, and 25	Humoral immune response, T-cell effector molecules, cytokines, chemokines, IFN-g-induced genes, epithelial cell polarity/morphogenesis, water transport, and cell adhesion
Wang *et al.* [21]	World J Gastroenterol 2005	Small int, rat	cDNA	Graft	Ischemia/reperfusion	Heterotopic segmental small bowel transplantation (sham operation) and controls; 1 h cold preservation/reperfusion	Cell adhesion, energetics
Schuurs *et al.* [22]	AJT 2004	Kidney, rat	Oligonucleotide	Graft	Brain death	Brain dead rats (normotensive or hypotensive)	Inflammation, fibrosis, repair, metabolism, transport, coagulation, cell cycling
Fujino *et al.* [23]	Liver Transplant 2004	Liver, rat	Oligonucleotide	Peripheral blood lymphocytes (PBL)	Tolerance	Tolerant versus syngeneic recipients	Signaling molecules, energetics, transcriptional regulation, cytokines, growth factors (and receptors), apoptosis, cell adhesion, and migration
Berg *et al.* [24]	Cell Transplant 2004	Pancreas, mouse	Oligonucleotide	Graft	Tolerance	Simultaneous bone marrow and pancreas transplant in nonobese diabetic mice	T-cell surface markers, cytotoxic T-cell molecules, cytokines, chemokines, and their receptors

Luckow *et al.* [25]	Euro J Immunol 2004	Heart, mouse	Oligonucleotide	Graft	Acute rejection (AR)	Fully mismatched MHC heart allografts in chemokine (C-C-motif) receptor-5 (CCR5) deficient mice and wild type	Decreased expression of metalloproteinase genes correlating with decreased vascular remodeling, intimal cellular infiltrate
Erickson *et al.* [26]	Transplantation 2003	Heart, rat	Oligonucleotide	Graft	AR	Heterotopic heart transplantation using ACI or Lewis donors and Lewis recipients, harvested on days 3, 5, and 7; cyclosporine versus tacrolimus	**Untreated allografts**: up-regulation of cytokines, chemokines, IFN-g, TGF, down-regulation of growth factor receptors, angiogenesis factors, lipid/drug metabolism; **treated allografts**: suppression of NO synthase, IFN-g; increased cell-cycle genes, lipid/steroid metabolism
Man *et al.* [27]	Liver Transplant 2003	Liver, rat	cDNA	Graft	Small for size graft injury	Allograft <30% of recipient liver weight versus whole grafts	Upregulation of cytokines, vasoconstriction, cell adhesion, apoptosis, vasopressin genes; downregulation of energy metabolism genes
Christopher *et al.* [28]	Phys Genom 2003	Heart, mouse	Oligonucleotide	Graft	Immune responses	Murine heart transplants in allogeneic (BALB/c to B6), syngeneic (B6 to B6), and alymphoid (BALB/c-RAG2–/– to B6-RAG1–/–) experimental groups	Upregulation of stress/injury response, cell metabolism, cell communication/defense, C1q, apo E; downregulation of creatine kinase, cytochrome C oxidase, decorin, lactate dehydrogenase myoglobin, actin, and troponin-I
Matsui *et al.* [29]	Phys Genom 2003	Heart, mouse	Oligonucleotide	Graft	Tolerance, AR	Cardiac allografts from BALB/c to C57BL/6 mice; tolerance induced by anti-CD80 and anti-CD86 monoclonal antibodies	Pro-inflammatory factors, IFNg-inducible cytokines and chemokines, apoptosis genes
Stegall *et al.* [30]	AJT 2002	Heart, rat	Oligonucleotide	Graft	AR	Brown Norway to Lewis heterotopic heart transplant, MHC mismatched	Inflammation, MHC antigens
Saiura *et al.* [31]	Transplantation 2002	Heart, mouse	Oligonucleotide	Graft	AR	Heterotopic allografts into IFN-gamma-deficient mice	Chemokines (JE/MCP-1, MIP1, PF4); notably absent were Mig, IP-10, and RANTES (CCL5) (IFN-g dependent)
Stegall *et al.* [32]	Transplantation 2002	Heart, rat	Oligonucleotide	Graft	Ischemia/reperfusion	Heterotopic brown Norway rat cardiac isografts removed on days 3, 5, and 7.	Upregulation of cytokines, adhesion molecules, integrins, and selectins; downregulation of cellular metabolism, mitochondrial function genes
Saiura *et al.* [33]	Transplant Int 2002	Heart, mouse	Oligonucleotide	Graft	Xenograft AR	Rat-to-mouse concordant xenograft cardiac transplantation	IFN-g inducible genes in both xenografts and allografts; Mac-1, cardionatrin and atrial natriuretic factors in xenografts
Amberger *et al.* [34]	Transplantation 2002	Heart, mouse	cDNA	Graft	Ischemia/reperfusion	Transplantation with 10 h cold ischemia or without cold ischemia, using native heart controls; studied at 2 min & 2, 12, 24 h post injury.	Immune modulators, receptor proteins, structural proteins, metabolism
Saiura *et al.* [35]	Transplantation 2001	Heart, mouse	Oligonucleotide	Graft	AR	Allografts (BALB/c to C3H/He), removed on days 1, 3, and 5	Primarily IFN-gamma, IFN-gamma inducible genes

*All studies were performed with species-specific microarrays with the exception of Aavik *et al.*, where cross-species hybridization of baboon samples were performed on human cDNA arrays.

Table 2. Relevant human microarray studies in solid organ transplantation, descriptive for array platform, tissue source, key genes, and associated biological processes.

Author	Journal	Organ	Platform	Tissue	Sample number	Phenotype	Selected key genes	Gene category
Hotchkiss *et al.* [36]	Transplantation 2006	Kidney	Oligonucleotide	Biopsy	22	CR	TGF-B, thrombospondin 1, PDGF, integrins, MMP7, C4B, properdin, VCAM1, Annexins, VEGF, EGF, FGF	Growth factors, fibrosis
Kurian *et al.* [37]	Transplantation 2005	Kidney	Oligonucleotide	Biopsy	12 (6 lap)	Laparascopic retrieval	HIF1α, HIF1B, TNF, TNFR, TGF-B, FGF, integrins, MMP, elastin, GHRH, and VEGF	Apoptosis, cell adhesion, cell signaling, cell growth/proliferation, immune response, inflammation, proteolysis, and stress response
Melk *et al.* [38]	Kidney Int 2005	Kidney	cDNA	Kidney cortex	20	Renal aging	NADH dehydrogenase, APO, kynureninase PAH, dynein, claudin-8, kinesin, MMP7, fibulin, tenascin, CSPG2, SERPINA3, immunoglobulins, somatostatin receptor, THY1, natriuretic peptide receptor, SLC solute transporter family	Energetics, oxidative stress, epithelial transport, hormone synthesis, solute transporters, extracellular matrix, cytoskeletal, and inflammatory response
Flechner *et al.* [39]	AJT 2004	Kidney	Oligonucleotide	Biopsy, PBL	32 (7AR)	AR	AIF, CD14, CD163, CD2, CD3D, CD48, CD53, chemokines, interleukins, C1q, immunoglobulins, INFg, TCR TNF, and HLA	Immune response, inflammation, cell cycling, and DNA metabolism
Horwitz *et al.* [40]	Circulation 2004	Heart	Oligonucleotide	PBL	21 (7 AR)	AR	UQCRB, BTF3, ST13, CUL4A, TERF2IP, ARRB2, NPEPPS, PIGB, APC, BCL7A, EDG4, IL 17R, PGF, NFAT5, BIRC1, LILRB3, TM6SF2, CFLAR, SOD2, SLC16A3, and SCD4	Cell-cycle regulation, immune response, apoptosis, and intracellular signaling
Kainz *et al.* [41]	AJT 2004	Kidney	cDNA	Biopsy	10 (5 CAD)	Donor source	Osteopontin, SOD2, RARRES1, chemokine ligand 1, antileukoproteinase, STAT1, CDH6, SPP1, SERPINA3, and GPX2	Oxidative stress counterbalancing
Flechner *et al.* [42]	AJT 2004	Kidney	Oligonucleotide	Biopsy	41	CR, drug effect	TGFB, TNFA, PDGF, ICAM, VCAM1, integrinB, MCP-1, CCR2, MPI-3B, MHC, MMP, TIMP1, RANTES, VEGF, collagen III, Angiotensin II receptor, thrombospondin, and fibronectin	Immune response, fibrosis, tissue remodeling, signal transduction, cell growth, and transcription
Mansfield and Sarwal [43]	AJT 2004	Kidney	cDNA	Biopsy	25	AR subtypes	HLA Class I and II, CD20, immunoglobulins, MIG, MIP-1, CCR5, CX3CR1, DARC, SCYB10, SCYA5, SCYA3, SCYA13, SCYA2, interleukins, (IL2RB, IL6R, IL16, 1L15R), DEFA1, DEFB1, SCYA2, SCYA5, MST1, STAT1, STAT6, CD69, MAL, NFATC3, Annexins, CASP10, PECAM1, and VCAM1	T- and B-cell activation, immune response, apoptosis, cell adhesion, cell cycling, HLA genes, and innate immunity
Hauser *et al.* [44]	Lab Invest 2004	Kidney	cDNA	Biopsy	36 [12 delayed graft function (DGF)]	DGF	Complement, LTF, NK4, VCAM1, interleukins, HLA, BCL6, GPX2,FBP1, PCK2, SORD, APOA4, CYP3A7, FABP1, APOM, CYP3A4, HIF1A, STAT1, TIMP1, ADAMTS1, TNFSF10, and CDC25B	Complement, immune response, energetics, hemostasis, cell cycling, and cell communication
Sarwal *et al.* [45]	NEJM 2003	Kidney	cDNA	Biopsy	67 (25 AR)	AR, CR, DT, and infection	TCR, HLA class II, HLA class I, immunoglobulins, lactotransferrin, chemokines, CD20, CD34, IGF1R, TNFR, MST1, NK4, duffy antigen/chemokine receptor, STAT1, TGFR1, granzyme A, perforin, IL2R, CD53, lymphotoxin, lymphotoxin R, NFKB1, CD59, IFNGR1, and annexins	Immune response (T and B cell), innate immunity, apoptosis, transcriptional regulation, and cell cycling

Donauer *et al.* [46]	Transplantation 2003	Kidney	cDNA	Biopsy	31 (13CR)	CR	AQP2, AQP3, lipoprotein lipase, PML-2, Napsin 1 precursor, Flotillin-1, Type IV collagenase, Hepatocte growth factor activator inhibitor, RIG-like 7–1, MECI-1, PGER, TEM8, MHC class I, C1s, and immunoglobulins	Energetics, cell signaling, cytoskeleton, cell adhesion, immune response, inflammation, and transcription
Scherer *et al.* [47]	Transplantation 2003	Kidney	Oligonucleotide	Biopsy	17 (9 CR)	CR	Keratin tumor suppressor candidate 7, OS9(APRIL), G-protein gamma7, protein/cell adhesion molecule-like, GRB2-associated binding protein 1, and PRLR	Cytoskeletal and cell differentiation/ proliferation
Gimino *et al.* [48]	Am J Resp Crit Care Med 2003	Lung	Oligonucleotide	Bronchoalveolar lavage	34 (7 AR)	AR	CD28, chemokines, CTLA-4, granzymes, perforin, Fas, immunoglobulin, IFNG, lymphotoxin B, perforin, TCR, CD84, complement, Lectin-like NK cell receptor, SLAM, STAT-4, and MASK	Immune response, apoptosis, transcription regulation, and cell signaling
Chua *et al.* [49]	AJT 2003	Kidney	cDNA	PBL	4	Anemia/AR	Hb-zeta, Hb-beta, Hb-alpha2, FOLR2, FOLR3, CAH1, immunoglobulins, GPX1, and lactotransferrin	Hemoglobin, folate, and iron biosynthesis
Inkinen *et al.* [50]	Transplant Proc 2002	Liver	cDNA	Biopsy (aspiration)	7 [4 cytomegalovirus (CMV), 3 AR]	AR, CMV	MHC class II, IL-2 receptor, caspase 1 and 3, granzymes, selectins, ICAM3, VCAM1, IFNg, IL-1, PDGFRB, AOC3, TGFB2, and TNF	Immune response, apoptosis
Zhang *et al.* [51]	Transplant Proc 2002	Kidney	Oligonucleotide	PBL	10	Stable transplant	CD80, interleukins, CD44, CD40L, CD40, VLA-5, LFA-1, TCR alpha, Lck, calcineurin, PKC, IFNG, TGF-B, TNF-alpha, TNFR1, G-CSFR, PDGF receptor, M-CSF 1, FGF R-1, and VEGF	Immune response, cytokines
Akalin *et al.* [52]	Transplantation 2001	Kidney	Oligonucleotide	Biopsy	10 (7 AR)	AR	HuMig, TCR RING4, ISGF-3, CD18	Immune response

findings confirmed a pathogenic role for intra-graft B cells (retrospective immunohistochemical staining of biopsy tissue for CD20), as potential antigen presenting cells for T cells [45,53]. Based on the findings in this study, B-cell infiltrates have become recognized as indicative of poor prognosis in kidney transplant rejection, and treatment with Rituximab, an anti-CD20 monoclonal antibody, has been initiated in some cases, as a potential therapy for a sub-group of patients with recalcitrant AR [54]. In contrast, the sub-group of patients with good outcome following AR episodes demonstrated up-regulated expression for genes involved in cell-cycling and cellular proliferation, but contained only minimal expression of genes responsible for lymphocyte infiltration and activation [45]. These results provide a strong basis for the clinical variability observed in AR. Therefore, stratification by molecular profiling may enable clinicians to predict treatment outcome, and individualize therapeutic interventions. Most importantly, the knowledge of these pathogenic mechanisms may lead to the discovery and implementation of novel therapeutic strategies.

Delayed graft function

Despite strict donor criteria and pretransplant organ inspection, postischemic renal failure occurs in 25% of deceased donor renal transplants and greatly impacts long-term graft survival. Hauser *et al.* [44] identified a set of 48 genes by microarray analysis, which classified deceased donor kidneys according to post-transplant course, identifying those grafts at risk for the development of postischemic acute renal failure. When compared with living donor kidneys, deceased donor kidney microarray profiles demonstrate increased expression of genes in the inflammatory cascade, including complement, cell adhesion molecules, and genes involved in apoptosis. This response to brain death, which has been demonstrated in animal models, may explain the propensity of deceased donor kidneys to develop delayed graft function, and may suggest the need to stratify management based on donor source.

Donor kidney age

A major constraint to transplantation is the shortage of available organs in the face of a constantly growing deceased donor waiting list [55]. As a result, use of extended criteria donors, including older donors, has become increasingly common [56]; however, donor age is a known correlate of allograft survival [57], particularly in renal transplantation. Gene expression analysis of kidneys of various ages performed by Melk *et al.* [38] supports clinical observations that significant heterogeneity exists

among donors, and age-related changes are not linearly associated with chronologic age. Changes in expression of genes related to tubular transport, cell cycling, energy metabolism, and response to oxidative stress occur over time, and likely affect the kidney's ability to withstand stress and injury. A set of 50 unique genes from this microarray analysis distinguished a subgroup of old kidneys (>70 years) with profiles similar to adult kidneys (31–46 years) from the remainder of the old kidneys. This unique data generated by microarray analysis may provide the foundation for a novel method by which clinicians can better use selected organs from older donors, thereby expanding the donor pool.

Chronic rejection

Microarray studies also carry the potential to uncover early triggers of immune and nonimmune injury that may drive the inexorable chain reaction resulting in *chronic graft injury and premature graft demise*. Chronic allograft injury, manifested as chronic allograft nephropathy in kidney, bronchiolitis obliterans in lung, 'vanishing bile duct syndrome' in liver, and coronary artery vasculopathy in heart transplantation, results from many potential etiologic factors, including both immune (e.g. rejection) and nonimmune mechanisms (e.g. hypertension, hyperlipidemia, and drug nephrotoxicity). No effective treatment options exist as mechanisms of chronic injury progression are unclear. While several studies have demonstrated the ability to use gene expression profiles to distinguish samples from patients with established chronic graft injury from those with stable allografts [45,46], they have not identified subsets within the population which correlate with clinical or histologic data. Similar to these prior studies, Hotchkiss *et al.* recently demonstrated a single cluster of 16 samples from patients with chronic allograft nephropathy, whose gene expression profiles showed upregulation of profibrotic and growth factors, as well as genes involved in the immune response [36]. The inability to stratify patients into subsets based on underlying mechanisms, including drug nephrotoxicity, may be attributable to late nonspecific injury patterns, regardless of the nature of the primary injury. A comparison of calcineurin inhibitor (CNI)-based versus CNI-free immunosuppression in renal transplant patients revealed more extensive chronic allograft injury in 2 years post-transplant in the CNI-based group, correlating with increased gene expression of pro-fibrotic and pro-inflammatory genes [42]. This suggests that sub-clinical molecular events relating to chronic graft injury occur over time and require early post-transplant serial sampling to discern etiology. The study of patients in the early stages of chronic injury, even prior to the development of histologic changes, may provide greater insight into the differences in the pathogenesis of chronic allograft injury. To address this question, Scherer *et al.* [47] analyzed early protocol renal allograft biopsies from 17 healthy recipients at 6 months post-transplantation in order to identify patients at risk for the development of CR. Changes in gene expression within the graft were detected in the early protocol biopsies of patients prior to the onset of overt clinical and histologic manifestations of CR. The pathways identified by this gene set were not previously known to be involved in allograft rejection and are, therefore, not obviously pathogenic. However, these findings lend credence to the hypothesis that early gene expression changes can signal the onset of injury and identify those patients at risk for CR and premature allograft failure.

Tolerance

Our laboratory has also been recently focusing on identifying noninvasive biomarkers for spontaneous graft acceptance in a select sub-group of immunocompetent transplant patients who have achieved successful immunosuppression minimization or discontinuation without adverse consequences. Tolerance is a rare occurrence, in which the recipient immune system ceases to recognize the graft as 'nonself', resulting in this ideal state of alloantigen acceptance. In current clinical practice, it is not possible to differentiate tolerant patients from the rest of the population, nor can we predict which patients have the potential to achieve tolerance. By using microarray technology to identify unique transcriptional profiles from patients who have successfully minimized or discontinued immunosuppressive medications (Sarwal *et al.*, unpublished data), we have been recently able to gain greater insight into the mechanisms underlying this rare event. Preliminary attempts to address the molecular basis of long-term rejection-free graft survival have suggested that immune hyporesponsiveness does not necessarily equate to immunological tolerance [51]. The identification of those patients who are truly immunologically tolerant remains a challenge to researchers. Microarray technology may offer novel ways to identify tolerant patients, and eventually use this information to promote or induce tolerance.

Microarrays: patient monitoring tools?

Microarray studies yield massive data output which requires cross-validation of significant data in large-scale, randomized trials. Therefore, in the near future, the most applicable use of microarrays for clinical monitoring lies in the field of biomarker discovery. Molecular profiling by DNA microarrays uses rapid whole-genome scanning

to generate sets of putative candidate disease *biomarkers or footprints* to create methods for diagnosing and monitoring patients.

Invasive tissue biomarkers on protocol biopsy interrogation may herald the onset of injury, although the search for markers specific for triggers of chronic graft injury remains elusive. *Panels* of tissue biomarkers can be used to differentiate prognostic groups of AR [43] which may then be used by the clinician to target patients for increased graft surveillance and those at risk for graft loss [45]. On the other hand, microarray studies have yielded *single gene* biomarkers, such as CD20, which now allow for unprecedented treatment stratification of AR [45]. Though informative, invasive biomarkers depend on the performance of a diagnostic biopsy, which is an invasive procedure with many associated risks.

Ideally, minimally or *noninvasive* post-transplant monitoring tools will be developed using blood, urine, or BAL samples, which will obviate the need for repeated invasive procedures for the diagnosis of intra-graft events. Using peripheral blood microarray analysis, Horwitz *et al.* [40] identified candidate biomarkers in gene expression profiles, which correlated with the presence of AR in cardiac transplant patients. Similar efforts in renal transplant patients found signatures in tissue, but were unable to identify biologically relevant peripheral blood biomarkers [39]. This may relate to small sample size, and may also result from underlying confounders in sample collection and processing that need further refinement. Discordance in signatures between tissue and blood microarrays also likely reflects the disparity between local and systemic immune responses, as well as the baseline differences in tissue-specific gene expression. Recently, investigators have demonstrated the ability to analyze blood or urine mRNA samples from renal transplant patients for single-gene biomarkers, and to use these results for the diagnosis of AR [58–63]. The success of these single-gene studies, as well as preliminary microarray data, raises the expectation that on-going, larger scale studies using peripheral blood microarray analysis are likely to yield promising results. The development of alternate genomic technologies, such as the TaqMan Low Density Arrays with up to 384 genes per array (http://www.appliedbiosystems.com/TLDA), is an excellent platform that can provide the rapid and more economical interrogation of selected informative genes that have been gleaned from previous genome-wide microarray experiments.

Microarray data analysis – a road block for real-time clinical monitoring

Microarray experiments generate large data sets that require a variety of data analysis software tools. Data analysis involves *five broad steps*: *data normalization* to compare expression levels, *data filtering* to eliminate genes expressed below a certain threshold, *pattern identification*, *literature mining*, and *gene family* and *pathway analysis*. Pattern identification of gene expression data can be either *unsupervised* (no prior knowledge of the data is used in the analysis) or *supervised* (prior knowledge of the data is used in the analysis). Popular methods of unsupervised analysis consist of Singular Value Decomposition (SVD) [64] and *clustering* algorithms, such as Hierarchical Clustering [65] and Self-Organizing Maps [66]. An SVD is used to determine if samples cluster because of an internal bias, such as batch run, and can be used to eliminate those genes associated with the bias. Hierarchical clustering is the most common algorithm used to group genes or samples based on their expression profile, creating a tree-like structure where the height of the branches is proportional to the distance between clusters. As hierarchical clustering only provides information on genes that are differentially regulated within the data set, Statistical Analysis of Microarrays [67] [statistical analysis of microarrays (SAM), www-stat.stanford.edu/~tibs/SAM/] was developed to identify genes with statistically significant changes in expression by producing a set of gene-specific t tests. SAM, available as a Microsoft Excel add-in, can be adapted to a broad range of experimental situations and can be applied to unsupervised and supervised analysis. An exciting application of supervised analysis is *class prediction* based on gene expression profiles, which can be used to identify the minimal set of genes that characterize a group of samples by phenotype. Two tools available for class prediction are predictive analysis of microarrays (PAM) [68] and ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Both tools run cross validation during the prediction process using a training set of arrays.

Once genes of interest have been identified, one must determine the *relevance* of these genes to the disease process being studied. One way to accomplish this is to conduct a literature search on currently available information. Search engines, such as PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), provide access to MEDLINE journals, as well as other general science and chemistry journals, and can be used to determine the interaction of individual genes. However, manual literature searches can be cumbersome because of the vast information available on well-known genes, as well as the abundance of less characterized genes for which functional information may be limited. Software tools, such as PathwayAssist (http://www.ariadnegenomics.com), Ingenuity Pathway Analysis (http://www.ingenuity.com/products/pathways_analysis.html), GeneSifter (http://www.vizxlabs.com/product_info.html), and GenMapp

[69] (http://www.genmapp.org/), aid the researcher in identifying the information, which is biologically relevant to the experimental dataset. These tools use genes of interest to build a pathway based on known interactions between these genes from the literature. The researcher can select a pathway to retrieve the journal citation of origin.

The final step in understanding the biological significance of differentially regulated genes in the data set is to identify their gene families and metabolic pathways. The Gene Ontology (GO) project [70] facilitated this step by standardizing gene family analysis through the organization of genes into functional families belonging to three main categories: molecular function, biological process, and cellular components. This innovative project provides a novel means for assigning biological relevance to gene expression data; however, it is important to recognize that the current knowledge base is expanding rapidly, and contains as yet incomplete information regarding different pathologic states. GoMiner [71] (http://discover.nci.nih.gov/gominer/index.jsp) provides quantitative and statistical information about the GO families that are over- or under- represented in the set of differentially expressed genes relative to the entire gene list. The genes of interest are linked to external data sources such as LocusLink, PubMed, NCBI's structured database, MedMiner, GeneCards, and KEGG. Expression Analysis Systemic Explorer [72] (EASE, http://david.niaid.nih.gov/david/ease.htm) is another tool that uses the GO categories, as well as custom and other public categories, to determine statistical gene enrichment. Both GoMiner and EASE provide links to the Kyoto Encyclopedia of Genes and Genomes [73] (KEGG, http://www.genome.ad.jp/kegg) to map gene function analysis to metabolic pathways. Databases, such as KEGG and MetaCyc [74] (http://www.metacyc.com), store information regarding metabolic and regulatory pathways for a wide range of species, thus allowing for cross-species analysis.

Data warehousing and reporting – essential requisites for collaborative data analysis

Currently much of the data generated by an individual or group is still unavailable to the scientific community or in a format that is unusable by other laboratories. With the continued discovery of new genes, scientific information is constantly changing. In addition, the field of transplant immunology is relatively new and information on the mechanisms of AR, CR, and tolerance or the effects of immunosuppressive medications is in its infancy. Making data sets publicly available will help the scientific community learn from each other, resulting in better patient care through the prediction of disease processes or individualization of treatment. Efforts are being made to develop improved analysis software and publicly available links to current updated internet-based information. Attempts to define a standard format for archiving expression data are underway, and a number of repositories now exist in order for data to be deposited into public or private gene expression databases such as Stanford Microarray Database, ExpressDB, The Gene Expression Database, and Gene Expression Omibus.

Limitations

Microarrays provide a high-throughput screening method of analyzing thousands of genes and discovering new biomarkers and metabolic pathways in different processes. However, some limitations exist that need to be considered.

1. Information regarding *proteins and metabolic processes* for corresponding genes is not provided by microarray analysis. Gene expression levels do not necessarily correlate with protein levels, as post-translational modifications cannot be measured by microarrays. Instead, one must currently use other methods, such as immunohistochemistry, to determine protein expression and localization. Proteomic research in transplantation and related fields is ongoing and may be a rich source of clinically relevant biomarkers [75,76], and the introduction of protein microarrays should provide a parallel high-throughput method for proteomic analysis. Similarly, metabonomic research, the study of metabolic responses of living systems to pathophysiological processes, can complement our understanding gained by transcriptional analyses.

2. Sampling variability, particular for renal transplant biopsies with differing amounts of cortex versus medulla represented in a sample, can greatly affect the pattern of gene expression of a sample. Publicly available gene lists specific for renal cortex and renal medulla have been generated and should be cross referenced to minimize false clustering of samples because of biopsy sampling rather than biological variability (http://genome-www5.stanford.edu/).

3. Weak overlap exists between gene lists from individual studies of similar phenotypes in transplantation (Table 2). The disparity among microarray data can be attributed to several factors:

- differences in microarray platform with differing gene sets;
- weak statistical power and small sample sizes;
- biological variance because of variability in patient characteristics;
- experimental variance, including lack of uniform protocols for study design, sample collection (lithium heparin versus TEMPUS versus PAX gene tubes),

RNA processing, and sample labeling and hybridization;
- different tools for data processing and statistical analysis: variable thresholds for data filtering, varying stringencies for false discovery rates, and statistical significance, different data analysis methods, including low level [RMA [77] (Robust Multichip Average) versus dChip (DNA chip analyzer http://www.biostat.harvard.edu/complab/dchip/) versus GCOS (GeneChip Operating Software), http://www.affymetrix.com/products/software/specific/gcos.affx] and high level signal analysis (SAM [67] versus PAM [68] versus BRB array tools).

A direct comparison study between the two most common platforms, cDNA microarrays and Affymetrix oligoarrays, found poor correlation of microarray results using 56 cancer cell lines [78]. Similar observations have been noted in solid organ transplantation, despite the growing numbers of datasets being generated. These issues have begun to be addressed, including a recent initiative to form a working group to perform meta-analyses of microarray studies across various laboratories and microarray platforms (http://www.cybernephrology.ualberta.ca/Banff/2005/highlights.htm). In addition, adoption of standards for sample collection and preparation, as well as the use of a broadly applicable, consistent platform, will increase the consistency and reproducibility of experimental results. The expectation is that identification of large panels of biomarkers as well as validation studies with large-scale, multicenter trials will address these issues.

4. Microarray technology is currently cost prohibitive for direct application to the clinical setting. In addition, expertise in the experimental technique as well as the bioinformatics analysis is required to perform the microarray assay itself, making it unrealistic to be universally adopted by clinical laboratories. Ideally, the data from these preliminary studies can be used to create a panel of biomarkers or a smaller scale array custom designed with sufficient sensitivity and specificity for diagnostic purposes. In addition, the development of multiplex platforms will allow for simultaneous analysis of multiple patient samples to make this a more cost effective tool.

Future directions

In reality, microarray technology is still several steps from implementation into clinical care of transplant recipients. Since the completion of the Human Genome Project, rapid advancements in genomics and related fields have had the potential to transform our approach to post-transplant patient monitoring. The ability for clinicians to individualize patient management is a major ambition of transplantation research. By providing information regarding individual risks of AR, DT, and CR, as well as the potential for developing tolerance, transcriptional profiling could enable clinicians to tailor immunosuppression and monitor patients based on the gene expression profiles. Although microarrays are far from ready for clinical implementation, great progress has been made toward the understanding of the underlying molecular mechanisms of transplantation, and the application of this information to improve patient monitoring. Ongoing and future multicenter collaborative studies, as well as meta-analyses of existing data, will enable the improvement and validation of the findings of the studies described in this review. In addition, incorporation of gene expression profiling into large-scale prospective clinical trials will allow for better correlation with clinical data (i.e. immunosuppression effects), improve prognostic capabilities, and enable researchers to delineate the variables involved in multifactorial processes such as chronic graft injury. With the introduction of this powerful genomic technique, these goals in solid organ transplantation which once seemed unattainable are developing into reality.

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