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Kidney Transplant Rejection and Tissue Injury by Gene Profiling of Biopsies and Peripheral Blood Lymphocytes

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Abstract

A major challenge for kidney transplantation is balancing the need for immunosuppression to prevent rejection, while minimizing drug-induced toxicities.

We used DNA microarrays (HG-U95Av2 GeneChips, Affymetrix) to determine gene expression profiles for kidney biopsies and peripheral blood lymphocytes (PBLs) in transplant patients including normal donor kidneys, well-functioning transplants without rejection, kidneys undergoing acute rejection, and transplants with renal dysfunction without rejection. We developed a data analysis schema based on expression signal determination, class comparison and prediction, hierarchical clustering, statistical power analysis and real-time quantitative PCR validation. We identified distinct gene expression signatures for both biopsies and PBLs that correlated significantly with each of the different classes of transplant patients. This is the most complete report to date using commercial arrays to identify unique expression signatures in transplant biopsies distinguishing acute rejection, acute dysfunction without rejection and well-functioning transplants with no rejection history. We demonstrate for the first time the successful application of high density DNA chip analysis of PBL as a diagnostic tool for transplantation. The significance of these results, if validated in a multicenter prospective trial, would be the establishment of a metric based on gene expression signatures for monitoring the immune status and immunosuppression of transplanted patients.

Keywords

DNA microarrays; gene expression; kidney; rejection; transplant

Introduction

Kidney transplantation has extended and improved the quality of life for the majority of patients with end stage renal disease. Most transplants involve genetically nonidentical

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donor-to-recipient combinations. As a consequence the immune response is a major impediment to successful graft survival, necessitating lifelong treatment with potent immunosuppressive drugs. These drugs suppress the host immune system in a nonspecific manner and have many side-effects including, but not limited to, increased risk of life-threatening infections and cancer. Another key point is that responses of the donor organ itself are also major contributors to post transplant events. Despite recent reductions in the incidence of acute rejection, chronic allograft nephropathy and immunosuppressive drug side-effects are still major causes of graft loss and patient morbidity. In this context, it is essential to further our understanding of the immune system and the transplanted organ to both immune and non-immune mechanisms of injury.

High-density microarray technology provides one means to measure the differential expression of hundreds to thousands of genes simultaneously. While its basic applications in gene discovery are well established, high-density microarrays also have promise as a clinical tool. For example, this technology has been used with different cancers to predict prognosis and response to therapy (1-3) and in multiple sclerosis to identify inflammatory genes in brain lesions (4). Several publications have examined gene expression in kidney transplant patients using quantitative PCR (5,6), and demonstrated that for a very small set of immunologically relevant gene transcripts good correlations with acute rejection and clinical outcomes were present. Studies in small animal transplant models using DNA microarrays supported the potential use of this technology in a clinical setting (7,8). A small study of kidney transplant patients with acute rejection demonstrated the up-regulation of four genes consistently and two transcripts down-regulated (9). Recently the experience using the Stanford Lymphochip cDNA glass slide array (10) with kidney transplant biopsies of 50 pediatric patients defined three different gene expression signatures for acute rejection that correlated with graft survival (11). Finally, a study using the Hu95Av2 Affymetrix GeneChip for kidney biopsies performed 6 months post transplant identified 10 genes for which expression correlated with the risk of developing chronic rejection defined by biopsy at 12 months post transplant (12).

In the present study we extended the work previously carried out in this field. We developed a data analysis strategy based on expression signal determination, class comparison and prediction, hierarchical clustering, statistical power analysis and real-time quantitative PCR validation. We determined gene expression profiles in biopsies obtained from normal kidneys at the time of their recovery for living donor transplantation, creating a unique control population for gene expression profiling of any renal disease including transplanted kidneys. This study includes a collection of profiles for transplant patients with normal graft function on full immunosuppression compared with transplant patients with biopsy-documented acute rejection. In addition, we provide the first gene expression profile information on patients with acute kidney transplant dysfunction who did not demonstrate evidence of histological acute rejection by biopsy. Finally, this is the first report of high-density DNA array gene expression profiles of peripheral blood lymphocytes (PBLs) from each of these classes of patients.

Hierarchical clustering of samples and statistical analysis of individual gene expression signals demonstrated significant differences in the profiles of biopsies and PBLs from patients with acute rejection and acute dysfunction without rejection as compared with normal donors and well-functioning transplant patients with no history of rejection. One implication of these results is that gene profiling of PBLs could be used as a minimally invasive surrogate marker for rejection and identify patients with acute dysfunction but without rejection. These data support the hypothesis that the gene expression profiles of PBLs can be used to dynamically monitor the state of the immune response to the transplant.

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Thus, it may be possible to determine the impact and adequacy of immunosuppression in individual patients at any time post transplant using DNA array technology.

Methods

Patients

Patients signed Cleveland Clinic Foundation-approved IRB consent forms. Kidney biopsies were obtained from nine living donor controls, seven recipients with histologically confirmed acute rejection, five recipients with renal dysfunction without rejection on biopsy, and 10 protocol biopsies carried out more than one year post transplant in patients with good transplant function and normal histology (Table 1). Peripheral blood lymphocytes were obtained from one living kidney donor and seven healthy volunteer blood donor controls, seven recipients with biopsy-proven acute rejection, eight recipients biopsied for renal dysfunction without rejection, and from nine of the 10 recipients who had protocol biopsies carried out more than 1 year post transplant (Table 1). It is important to emphasize that all the acute rejection profiles of transplant biopsies and PBLs are matched to the same patients for all samples. For example, AR3 PBLs are from the patient of biopsy AR3. Evaluation of renal function for living donors included creatinine clearance, protein excretion and renal imaging with ultrasound and angiography. Acute rejection episodes were Banff criteria scored (13) and confirmed by response to anti-rejection therapy. Patients with clinical or laboratory evidence of CMV or other infections were excluded. Immunosuppression comprised a calcineurin inhibitor or sirolimus, with mycophenolate mofetil and steroids. Control biopsies were obtained from the cortex of diuresing kidneys during open-donor nephrectomies. Transplant biopsies were obtained under ultrasound guidance by spring-loaded 15-gauge needles (ASAP Automatic Biopsy, Microvasive, Watertown, MA). Cores went immediately into 1.5 mL of RNALater (Ambion, Austin, TX), and −80°C freezers within 4 h. Peripheral blood (20 mL) was obtained before biopsy, placed on ice and mononuclear cells were isolated within 1 h by density-gradient centrifugation and stored in RNALater at −80°C.

RNA isolation

Frozen biopsy specimens were thawed, poured into 2-mL tissue grinders with 1 mL of Trizol (Invitrogen, Carlsbad, CA) and manually homogenized. Frozen PBLs were thawed and disrupted in 1 mL of Trizol using a 21-gauge needle. Total RNA was isolated from homogenates using chloroform: isopropanol and further purified using an RNeasy column (Qiagen, Valencia, CA) and DNAse-treated (DNA-free, Ambion) to remove genomic DNA. RNA quality was confirmed by electropherograms using an Agilent 2100 BioAnalyzer (Palo Alto, CA). Total RNA yields from 14 consecutive 15-gauge needle biopsies were 14.9 ± 3.9 μG.

Microarray analysis

For tissue biopsies, standard Affymetrix GeneChip (Santa Clara, CA) protocols were used [affymetrix.com (14)]. RNA extracted from PBLs underwent one additional round of RNA amplification owing to limited RNA yields in the early samples of the study. Amplification was carried out starting with 100 nG of total RNA using the Ambion MEGAscript™ aRNA Amplification Kit following the manufacturer's protocols. All labeled samples were hybridized to HG-U95Av2 GeneChip arrays. GeneChip data were analyzed using Microarray Suite 5.0 (MAS 5.0, Affymetrix) and DNA Chip Analyzer (dChip) (15,16) software using the PM only model. ‘Present’ and ‘Absent’ calls were determined with MAS 5.0. The dChip software used all the Affymetrix.CEL files generated in this study as a training set. BRB Array-Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) was used to perform hierarchical clustering and class prediction. Statistically significant changes in gene

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expression were measured with Significance Analysis of MicroArrays (SAM v1.3; 17). Delta values were chosen to minimize the median false discovery rate (FDR) at a level less than one false discovery per gene list. Two additional methods were used to filter the gene list. First, we applied the limit fold change model, which systematically bins genes by signal intensity; those genes within the top 10% of the highest fold changes for each bin were selected (18). Second, MAS 5.0 Present/Absent calls were used to filter the list; we required the majority of calls in the up-regulated group to be 'Present'.

Real-time quantitative PCR (Q-PCR)

Q-PCR was performed on 15 genes selected for relatively large fold-changes from the list of 65 genes shown in Figure 3B using predesigned primer and probe sets from the Assays-on-Demand Genomic Assays (12 genes) and Assays-by-Design service (three genes) (Applied Biosystems, Foster City, CA). Each assay consisted of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB probe. The endogenous control, 18S rRNA, was detected with a VIC™ dye-labeled TaqMan® MGB probe. Briefly, cDNA was transcribed from 100 nG total RNA using the ABI cDNA Archive kit (Applied Biosystems). Nine μL of the cDNA reaction was added to 11 μL of Platinum® Quantitative PCR SuperMix-UDG PCR reagent (Invitrogen, Carlsbad, CA) and PCR performed on an ABI Prism 7900HT (Applied Biosystems). All amplifications were carried out in triplicate and threshold cycle (C_t) scores were averaged for calculations of relative expression values. The C_t scores for genes of interest were normalized against C_t scores for the corresponding 18S rRNA control. Relative expression was determined by the following calculation where the amount of target is normalized to an endogenous reference (18S rRNA) and relative to an arbitrary calibrator (the reference class of patients used in the comparison):

$$\text{Relative Expression}=2^{\Delta\Delta Ct}, \text{where}^{\Delta\Delta Ct}=\left(^{\Delta Ct}\text{of experimental group}\right)-\left(^{\Delta Ct}\text{of calibrator group}\right)$$

Power calculations

Power calculations for application to microarray experiments has been attempted by several research groups (Simon, 2003; Zien, 2003). The basic premise is to determine the variability for measurements of gene expression by standard deviation of the results of multiple samples. While there is not general agreement on a single best method to perform these calculations, the data we had collected to date provided us with real data upon which to make estimates of variability. Variability for a measurement is described in terms of the standard deviation and is the key experimental metric for sample size calculations. In this context, the measurement is the mean signal intensity measured for each gene's probe set on the GeneChip. The variance value (s) was based on the median $\log_2$ transformed signal intensities derived from our data on more than 30 experiments using the GeneChips on either transplant biopsy or PBL samples. The next step is to set values for an acceptable alpha error (false-positive rate), beta error (false-negative) and the delta (minimal detectable change that will be confidently determined). We used values of 0.001 (alpha; a), beta (b) of 0.8 and a minimal detectable fold change of 2 (delta; d).

Calculations were performed using the following equation:

$$n=4(Z\alpha/2+Z\beta)^2/(\delta/\sigma)$$

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Web site data

All the.cel files for the Affymetrix GeneChips used in these studies are available to the public at our TSRI DNA Array Core in MIAME compliant format (URL: www.scripps.edu/services/dna_array/). We also provide at this site a series of annotated gene lists including literature references.

Results

Gene expression analysis of kidney transplant biopsies

To define gene expression profiles in kidney transplant patients we assembled a series of biopsy samples from normal living kidney donors (C) and several classes of patients including well-functioning kidneys more than 1-year post transplant (TX), biopsy-confirmed acute rejection (AR), and acute renal dysfunction without rejection (NR) (Table 1). After signal expression determination using dChip, we used hierarchical clustering of samples based on their individual gene expression profiles as a tool to examine the relationships between experimental groups. Clustering of (C), (TX), and (AR) indicates that each group is distinct with respect to their gene expression profiles (Figure 1A). It is important to note that this cluster analysis was performed using an unsupervised data set, essentially all genes called as Present on at least one chip (8320 genes; 66% of the probe sets on the chip). The purpose of an unsupervised clustering is to avoid introduction of bias based on preclassification of gene expression by sample type.

This clustering pattern demonstrates that gene expression defines distinct groups of transplant patient biopsies, specifically separating acute rejection from well-functioning transplants and from normal kidneys unex-posed to immunosuppression. Therefore, we performed a class comparison analysis between acute rejection and biopsies from fully immunosuppressed patients with good graft function (AR vs. TX) (Figure 1B). This comparison identified the subset of differentially expressed genes, up-and down-regulated, that define acute rejection. We also compared gene expression in healthy donor kidneys with that of transplant recipients with good graft function and full immunosuppression (TX vs. C). This comparison identifies gene expression profiles that define the impact of transplantation and immunosuppression on a normal donor kidney.

We determined significant changes in gene expression comparing biopsies of acute rejection to those of the stable transplants (AR vs. TX). Using SAM we identified 96 up-regulated and 619 down-regulated genes (median FDR $<0.14\%$ per comparison). We created an annotated gene list based on a literature search (Figure 1B). These results show that genes involved in immune and inflammatory responses represent the dominant category of up-regulated genes in acute rejection (44 of 96 genes; 46%). Interestingly, a large number of the genes down-regulated in acute rejection are involved in different categories of basic cellular metabolism that might reflect the impact of rejection and immunosuppressive drug-mediated tissue injury on the kidney.

Next we compared the biopsies of the fully immunosuppressed recipients with normal graft function with those from normal living donors (TX vs. C). We identified and classified 612 up-regulated and 28 down-regulated genes (median FDR $<0.16\%$; Figure 1B and supplemental data). Even a year or more post transplant, well-functioning kidneys had a distinct gene profile compared with the normal donor controls. Possible explanations for these differences in gene expression include an underlying subclinical immune response, the impact of post transplant drug therapies, compensatory physiological changes in a single kidney, and tissue responses by the transplanted kidney to these injury pathways. For example, genes that are up-regulated and define the differences between the transplanted and normal donor kidneys include 45 genes classified with cell growth and regulation, 47 with

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protein metabolism, 35 as structural and 66 as transcription factors or other gene expression regulators.

An important question is the nature of the immune response in well-functioning kidney transplants without clinical or biopsy evidence of rejection. There are 45 up-regulated genes classified as immune/inflammatory in well-functioning transplants (Figure 1B) compared with the normal donor control kidneys (7.3% of 619). Interestingly, this gene set does not overlap with the list of immune/inflammatory genes significantly increased when acute rejection biopsies are compared with the well-functioning transplants (AR vs. TX; Figure 1C). The largest group within the immune/inflammatory genes up-regulated in the well-functioning transplants is histocompatibility antigens consistent with the hypothesis of an ongoing but low-grade immune response or some form of tissue injury resulting in cytokine-mediated induction of MHC molecule expression.

A common clinical problem is acute renal dysfunction resulting from nonimmune-mediated injury of the transplant (i.e. drug toxicity and ischemic injury). Roughly 50% of the biopsies carried out during this study for acute renal dysfunction did not reveal acute rejection by histology. Therefore, we examined the differential gene expression profiles of patients with acute renal transplant dysfunction in which the biopsy histology did not demonstrate rejection (NR). Unsupervised hierarchical clustering demonstrated a good separation of the well-functioning transplants (TX) from the profiles of kidneys with acute dysfunction (AR and NR; Figure 2A). However, it was not possible to distinguish the AR and NR biopsy groups.

We hypothesized that there were at least two predominant gene groups within the expression profiles of the AR and NR biopsies, one comprised of genes related directly to the acute immune-mediated rejection and another representing genes common to tissue injury and kidney dysfunction. If the second group of injury-associated genes was much larger, then it could explain the inability of unsupervised cluster analysis to separate the AR from NR biopsies. Therefore, we performed a two-class comparison analysis in BRB ArrayTools of the gene expression profiles comparing AR with NR. This gave us 65 genes at a 0.001 significance level. The results of a three-class comparison analysis comparing AR with NR with TX was 3550 genes at the 0.001 significance level; consistent with our hypothesis that the set of genes associated with kidney injury/dysfunction is indeed larger then the gene list associated with acute rejection. Thus, we performed a supervised hierarchical clustering using just the 65 genes identified as distinguishing AR from NR (Figure 2B). The supervised approach gives a clear separation of all three clinical groups. By functional class, the 65 genes identified as distinguishing AR from NR contain 12 genes associated with immune/inflammation responses (17%), seven of which are also in the immune/inflammation group of 44 genes up-regulated in the profiles of acute rejection biopsies compared with well-functioning transplants (AR vs. TX; Figure 1C).

Gene expression analysis of peripheral blood lymphocytes

To assess the impact of immunosuppression and acute rejection, PBLs were collected from: a control group of healthy, nonimmunosuppressed blood donors (C), immunosuppressed kidney transplant recipients with well-functioning kidneys and no history of rejection (TX), and immunosuppressed kidney transplant recipients with acute renal dysfunction documented by biopsy to be owing to either rejection (AR), or non-immune-mediated pathology (NR). Unsupervised hierarchical clustering analysis of the array data was performed (Figure 3A,B).

These data show that PBLs from immunosuppressed transplant patients with well-functioning kidneys (TX) cluster separately (Figure 3A). Peripheral blood lymphocytes from

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transplant patients with renal dysfunction owing to either AR or without biopsy evidence of rejection (NR) cluster predominantly into separate groups. However, AR5 clusters with the NR PBLs, and NR2 clusters with the AR PBLs. These exceptions suggest the possibility that some level of acute renal dysfunction can be immune-mediated and yet fall below the level detected by the biopsy. However, a much larger data set will be required to test this hypothesis. Unsupervised clustering of the healthy donor PBLs (C) and PBLs from immunosuppressed kidney transplant recipients with well-functioning kidneys (TX) demonstrates distinct separation of the PBL profiles from nonimmunosuppressed donors (Figure 3B). Nonetheless, for reasons that are unclear, samples C5 and C1 cluster independently from the other control PBL samples with a low correlation branch to the cluster of PBL from immunosuppressed patients with well-functioning transplants.

We created an annotated gene list based on a literature search (Figure 3C). One striking difference for the PBLs, in comparison with the transplant biopsies (Figure 1B), is that genes classified as immune/inflammatory are not a dominant category, particularly in patients with biopsy-proven AR. However, the PBL profiles for patients with well-functioning transplants on full immunosuppression compared with normal blood donors (TX vs. C) reveal a significant up-regulation of genes classified as immune/inflammatory (13; 8%), cell growth and regulation (13; 8%), protein metabolism (24; 15%) and transcription factors/regulators of gene expression (17; 11%). Interestingly, none of the 13 immune/inflammatory genes up-regulated in the profiles of PBL from patients with well-functioning transplants (TX vs. C) are identified in the list of 45 such genes identified in the same comparison based on the biopsy data (Figure 1B). Analysis of the specific genes in the four functional classes (Figure 3D) up-regulated in PBLs from patients with acute rejection compared with well-functioning transplants (AR vs. TX) and PBLs from patients with well-functioning transplants compared with normal blood donors (TX vs. C) reveals that there are only three genes that overlap with the genes up-regulated in the biopsies (Unigene #: Hs. 183037, Hs. 18192, Hs. 75248). Thus, it is evident that the gene expression profiles of PBLs are very different than those of the biopsies in the various classes of transplant patients.

Predicting clinical status of kidney transplants from gene expression profiles

A test of our hypothesis that distinct gene expression profiles correlate with clinically and biopsy-defined phenotypes in kidney transplantation is to demonstrate successful use of class prediction tools to correctly separate the phenotypes. We used six class predictors implemented in BRB ArrayTools for determining to which of two or more predefined groups an unknown sample belongs. If class prediction results of PBLs gene expression profiles correlate with clinical phenotypes, then monitoring of patient status would be possible with blood sampling. Thus, we tested all of the six class prediction algorithms currently available in BRB Array Tools for both biopsy and PBLs profiles (Table 2).

In the comparison of TX vs. AR, the performance of a ‘leave-one-out’ cross-validation correctly classified from 94 to 100% of the biopsies and 93% of the PBL gene expression profiles. Class prediction results for the comparison of PBLs and biopsy profiles of acute rejection with non-rejection patients (AR vs. NR) were generally unsatisfactory. These results match the problems we encountered in the unsupervised clustering of these data (Figure 2A). In contrast, comparison of the samples from well-functioning transplants with those from the non-rejection patients (TX vs. NR) correctly classified 100% of the biopsies and 94% of the PBL profiles. Moreover, class prediction comparing well-functioning transplants with the combined AR/NR group resulted in 100% correct classifications for both biopsy and PBL data. These results are consistent with the hierarchical clustering shown above (Figure 2). Finally, class prediction for normal donor kidneys compared with well-functioning kidney transplants (C vs. TX) was successful in classifying 100% of the biopsies and 88–94% of the PBL gene expression profiles. Therefore, it is evident that gene

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expression in well-functioning kidney transplants is not the same as normal kidneys and these differences may help to identify the impacts of immunosuppressive drugs, immunity and transplant surgery. These results also support the hypothesis that the impact of immunosuppression and transplantation may be profiled successfully in the peripheral blood compartment.

Validation of GeneChip data

One method of data validation is quantitative PCR. We chose 15 genes from the list of 62 classifying AR vs. NR biopsies (Figure 2C) for validation by quantitative PCR (Table 3). Three biopsies from each of the four clinical classes were chosen based on adequate material for analysis. We compared all the clinical classes for all the comparisons involving these 15 genes where SAM analysis indicated a significant change was present. These results demonstrated agreement in 20 of 21 comparisons with respect to the direction of gene expression change at a highly significant level ($p = 0.0001$). In general, fold changes determined by quantitative PCR were greater than those detected by GeneChip data analysis. These results suggest that the dynamic range of current GeneChip technology is relatively low, though the direction of expression changes are accurate. Thus, Q-PCR and similar quantitative measures of RNA expression are important and complementary tools.

Another important aspect of validating data for gene expression signatures correlating with specific patient groups is the appropriateness of the sample sizes studied. While there is not general agreement on a single best method for statistical power calculations in microarray experiments the development of formulas has been attempted by several research groups (19). We performed a power analysis of this study using our sample sizes and variance based on the median standard deviation of gene expression measurements. Our power to compare the expression profiles of acute rejection (AR) with the nonrejection and well-functioning patient groups (NR, TX) is 86% and 99%, respectively, for the biopsy data and 97% and 99%, for the PBLs. Thus, these data do reveal that our gene expression signatures correlate significantly with specific patient groups.

Discussion

The ability to measure gene expression profiles in kidney transplantation allows us to test several hypotheses that will directly impact on clinical practice. Currently, there is no objective measure for determining the adequacy of immunosuppression, and no objective way of predicting an individual patient's response to therapy. Clinical practice is based on experience with large populations of patients that are empirically individualized by transplant physicians to take into account factors identified as unique to a given patient such as an early acute rejection episode, evidence of drug toxicity, and serial measurements of renal function. There is also a constant pressure to reduce or eliminate drugs to avoid long-term toxicity and cost. Therefore, if gene expression profiling identifies a signature for acute rejection, then a patient on any given immunosuppressive regime could be monitored for that signature as a measure of the adequacy of immunosuppression. In turn, decisions to reduce or eliminate immunosuppressive drugs could be made with a strategy to safely monitor the results before clinically apparent changes in kidney function occur. It may also be possible to improve the safety of new immunosuppressive drugs, particularly in establishing dose responses, and testing the efficacy of combining new agents with existing drug regimes.

The data presented in this study reporting an acute rejection signature for both PBLs and transplant biopsies supports the hypothesis that a prospective approach to monitoring molecular changes in transplant patients could also be used to predict acute rejection. If determining the adequacy of immunosuppression and predicting rejection could be carried

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out with PBLs alone, then the potential for a minimally invasive monitoring strategy would be realized. Moreover, an important goal of molecular medicine is to develop tools that effectively allow physicians to individualize therapy. However, we understand that an adequately powered prospective clinical trial would be required to test this hypothesis developed with our data and validate such a diagnostic strategy.

Another hypothesis that should be tested is that gene expression signatures can be used to predict chronic allograft nephropathy early enough to alter therapy. In this context, subclinical rejection identified in early protocol biopsies supports the hypothesis that rejection can be present long before evidence of clinical kidney dysfunction emerges (20-23). The results of Scherer et al. support this hypothesis, indicating that gene expression profiles of protocol biopsies at 6 months could predict biopsy changes of chronic rejection at 12 months (12). Therefore, a major question is whether there is a continuum between subclinical acute rejection and chronic allograft nephropathy that represents the mechanistic link between the events determining rejection, tissue injury, and repair. If such a continuum can be defined in molecular terms, then the potential of therapeutic interventions can be tested.

There remain a number of problems with the present approach that must be considered. The heterogeneity of our patient populations, differences in immunosuppressive therapy, and different degrees of rejection all contribute to biological variability in gene expression profiles that will reduce the number of statistically significant genes we have identified. Thus, while our statistical power analysis demonstrates that our group sizes are sufficient to support the conclusions we have made regarding the significance of expression signatures, it does not mean that all the genes that play a significant role in transplantation have been identified. Moreover, much larger sample sizes of patients are required to draw conclusions regarding the correlations between these gene expression signatures and clinical outcomes such as response to antirejection therapy, long-term graft function and survival. In addition, a limitation of the current microarray technology is that the sensitivity and specificity of gene expression profiling is difficult to determine objectively when thousands of genes are studied simultaneously. Of course, the HG-U95Av2 GeneChip used here represents conservatively one-third of what is now considered the full human genome and the technology has already advanced to the latest version, the HG-U133 chip set. Thus, for all these reasons it is certain that many important genes are not included in our lists. One way to address these limitations would be to design the large and prospective trial discussed above and use the latest microarrays with a more complete representation of the human transcriptosome as well as other technologies such as quantitative PCR to validate and extend these studies.

While the clinical impact of gene expression signatures that can predict rejection and monitor immunosuppression is clear, the potential contributions to our basic understanding of transplantation biology are also important to consider. Thus, the ultimate objective of gene expression profiling is to identify specific genes and associate these with specific pathways mediating cellular mechanisms of rejection, tissue injury and repair, immunosuppression and tolerance. Therefore, we have taken care to provide lists organized by both function and specific gene names for all our significant group comparisons. We also have placed all our data files in MIAME format at our web site for public access. However, a key point is that the fields of bioinformatics and systems biology are still in their infancy with respect to taking specific gene sets and reliably establishing biological pathways. Therefore, we have concentrated on establishing the validity of our first hypothesis that gene signatures can be correlated with well characterized clinical phenotypes all established by the current gold standard of a transplant biopsy. Of course, in all these sets there are genes that we recognize and can find literature regarding their biological function and correlation

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with immune responses and transplantation models of various types. These are provided with annotations at our web site. But there are also many genes and pathways that are presently not fully understood or characterized and some are likely to be misunderstood at the current time.

How are lymphocytes in the peripheral lymphoid compartment influenced by events that occur within the kidney transplant such as antigen recognition and the signaling events responsible for allo-immune activation? Our results demonstrate that PBL gene expression profiles in acute rejection are distinctly different from those of normal controls and from patients with well-functioning transplants. Therefore, acute rejection does influence the gene expression profile of the circulating lymphocyte pool. Moreover, despite the fact that surprisingly we found very little common gene expression between PBLs and kidney biopsies, we did identify a large number of lymphocyte-specific genes in the kidney tissue. One interpretation is that there are compartment-specific differences between the PBLs in the circulation and the subset of lymphocytes that are activated and recruited to the transplant kidney during acute rejection. The significance of these results in the context of monitoring patients after transplantation is that they may explain the failure of more than a decade of work testing PBLs for an array of activation antigens based on findings in rejecting allografts and other immune models. In other words, the activated lymphocytes infiltrating the rejecting allograft are a distinct population compared with the circulating PBL pool. It is possible that the gene expression profile of the PBLs represents the adequacy of immunosuppression such that the rejecting patients reflect the profile of inadequate immunosuppression as compared with the PBLs sampled from patients with well-functioning transplants. Perhaps future drug therapies could be advanced by targeting the genes that are up-regulated in these PBL profiles. Nonetheless, our results do demonstrate that there is a distinct gene expression profile in the PBL pool that correlates with acute rejection and immunosuppression. If these results can be confirmed in a large, prospective trial it would support the use of such profiles as a minimally invasive monitoring strategy for the immunological status of the graft and support the potential of using them to monitor the adequacy of immunosuppression.

One limitation to consider is that we purified PBLs for analysis using a density gradient and performed one round of amplification of the mRNA before the standard labeling procedure. It is known that such physical handling of PBLs can result in *ex vivo* cell activation and gene induction. Secondly, amplification of RNA transcripts can also bias gene expression measurements. We were consistent in using the same protocol for all PBLs samples studied, both for amplification and processing, such that there should be no class-specific bias in the expression profiles obtained. However, recently several new technologies have been developed that will eliminate this issue by allowing investigators to draw peripheral blood samples directly into preservation solutions that instantly capture the transcriptosome at that time of the draw. Finally with respect to the possibility of RNA amplification introducing bias, it is important to note that a number of studies have been carried out demonstrating consistent gene expression profiles carried out with two and in some instances three rounds of amplification (24,25).

Given that chronic allograft nephropathy is a major cause of transplant dysfunction and loss, another question is the status of the well-functioning kidney transplant. Our results demonstrate that despite good graft function in this group there is a distinct up-regulation of inflammatory/immune response genes in both biopsies and PBLs. One possibility is that there is a continuum of immune activation that defines the status of a transplant at any given time. This activation state is influenced by factors such as the adequacy of immunosuppression, genetics, and environment. We believe that the long-term function of the transplanted kidney is determined by the intersecting effects of both recipient and donor

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genetics. Namely, the nature of the recipient's immune response integrated with the donor organ's response to tissue injury, including the impact of nephrotoxic drugs. Theoretically, it should be possible to distinguish genes expressed by the donor organ from genes expressed by the host's infiltrating cells using techniques such as laser capture microdissection.

In conclusion, we have developed a strategy for integrating a number of gene expression profiling and supervised and unsupervised statistical tools to generate lists of genes that represent at least parts of the complex biological pathways involved in transplantation biology. In this context, we acknowledge the fact that at the present time the function of only a minority of the human genome is documented. As the knowledge base that can be accessed through bioinformatics grows to better define cellular pathways and regulatory networks, these gene lists linked to well-defined clinical events in transplantation will provide additional opportunities to advance our understanding of the basic biology of transplantation and identify new targets for therapeutics.

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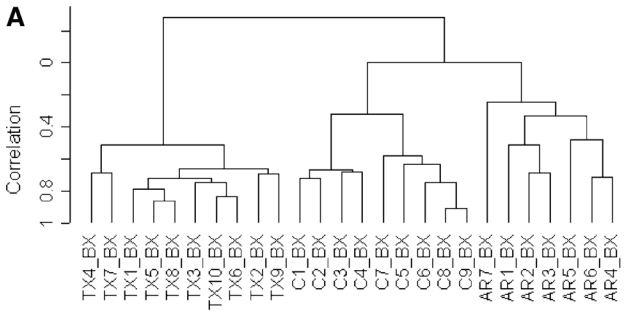
A



B

Functional Classes	AR vs. TX Up regulated	AR vs. TX Down regulated	TX vs. C Up regulated	TX vs. C Down regulated
Amino acid (and related products) metabolism	1	41	11	0
Apoptosis	0	4	9	0
Carbohydrate metabolism	1	34	18	0
Cell Adhesion	0	5	10	0
Cell growth/regulation	4	35	45	4
DNA repair/metabolism	2	10	17	1
Drug elimination/metabo	0	8	4	2
Energy (electron transport, ox phos and associated functions)	0	33	7	0
Extracellular matrix	2	2	4	0
Fatty acid / lipid metabolism	3	29	15	0
Glycosylation/glycosylase	0	5	10	0
Immune/inflammation response	44	5	45	1
Intracellular signaling	8	61	68	1
Miscellaneous	5	19	22	2
Neuro-related	1	6	0	0
Not defined	3	157	114	2
Oxidative stress	0	5	1	0
Protein synthesis/metabolism	10	37	47	10
Structural	4	19	35	2
Transcription factors and gene expression regulators	5	28	66	2
Transport	1	54	32	1
Vesicle trafficking, endo/exocytosis	2	22	32	0
Total	**96**	**619**	**612**	**28**

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C

	AR vs TX; up in AR			Tx vs C; up in TX
Unigene ID	**Gene**		**Unigene ID**	**Gene**
Hs.76364	allograft inflammatory factor 1		Hs.169610	CD44 antigen (homing function and Indian blood group system)
Hs.75627	CD14 antigen		Hs.84298	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
Hs.74076	CD163 antigen		Hs.1244	CD9 antigen (p24)
Hs.89476	CD2 antigen (p50), sheep red blood cell receptor		Hs.78913	chemokine (C-X3-C motif) receptor 1
Hs.95327	CD3D antigen, delta polypeptide (TiT3 complex)		Hs.385710	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
Hs.901	CD48 antigen (B-cell membrane protein)		Hs.77367	chemokine (C-X-C motif) ligand 9
Hs.82212	CD53 antigen		Hs.169756	complement component 1, s subcomponent
Hs.85258	CD8 antigen, alpha polypeptide (p32)		Hs.78065	complement component 7
Hs.276770	CDW52 antigen (CAMPATH-1 antigen)		Hs.201673	cornichon-like
Hs.241392	chemokine (C-C motif) ligand 5 (RANTES)		Hs.62661	guanylate binding protein 1, interferon-inducible, 67kDa
Hs.89414	chemokine (C-X-C motif) receptor 4		Hs.278568	H factor (complement)-like 1
Hs.8986	complement component 1, q subcomponent, beta polypeptide		Hs.73885	HLA-G histocompatibility antigen, class I, G
Hs.155597	D component of complement (adipsin)		Hs.300697	immunoglobulin heavy constant gamma 3 (G3m marker)
Hs.205353	ectonucleoside triphosphate diphosphohydrolase 1		Hs.153261	immunoglobulin heavy constant mu
Hs.73946	endothelial cell growth factor 1 (platelet-derived)		Hs.76325	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides
Hs.433300	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide		Hs.406565	immunoglobulin kappa constant
Hs.77424	Fc fragment of IgG, high affinity Ia, receptor for (CD64)		Hs.405944	immunoglobulin lambda locus
Hs.176663	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)		Hs.155530	interferon, gamma-inducible protein 16
Hs.90708	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)		Hs.362807	interleukin 7 receptor
Hs.171862	guanylate binding protein 2, interferon-inducible		Hs.385838	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1
Hs.18571	Human interferon-inducible RNA-dependent protein kinase (Pkr) gene		Hs.76507	LPS-induced TNF-alpha factor
Hs.104119	Human renal cell carcinoma antigen RAGE-4 mRNA		Hs.153563	lymphocyte antigen 75
Hs.8904	Ig superfamily protein		Hs.181244	major histocompatibility complex, class I, A
Hs.83968	integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)		Hs.277477	major histocompatibility complex, class I, C
Hs.366	interferon induced transmembrane protein 1 (9-27)		Hs.110309	major histocompatibility complex, class I, F
Hs.183487	interferon stimulated gene 20kDa		Hs.914	major histocompatibility complex, class II, DP alpha 1
Hs.14623	interferon, gamma-inducible protein 30		Hs.814	major histocompatibility complex, class II, DP beta 1
Hs.327	interleukin 10 receptor, alpha		Hs.198253	major histocompatibility complex, class II, DQ alpha 1
Hs.173936	interleukin 10 receptor, beta		Hs.76807	major histocompatibility complex, class II, DR alpha
Hs.75545	interleukin 4 receptor		Hs.375570	major histocompatibility complex, class II, DR beta 1
Hs.105938	lactotransferrin		Hs.318720	major histocompatibility complex, class II, DR beta 4
Hs.81337	lectin, galactoside-binding, soluble, 9 (galectin 9)		Hs.83532	membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)
Hs.67846	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4		Hs.943	natural killer cell transcript 4
Hs.380427	leukocyte specific transcript 1		Hs.54483	N-myc (and STAT) interactor
Hs.204238	lipocalin 2 (oncogene 24p3)		Hs.394389	peptidylprolyl isomerase B (cyclophilin B)
Hs.381099	lymphocyte cytosolic protein 1 (L-plastin)		Hs.18571	protein kinase, interferon-inducible double stranded RNA dependent activator
Hs.1765	lymphocyte-specific protein tyrosine kinase		Hs.75445	SPARC-like 1 (mast9, hevin)
Hs.110309	major histocompatibility complex, class I, F		Hs.389371	stromal cell derived factor receptor 1
Hs.184018	MD-1, RP105-associated		Hs.153026	SWAP-70 protein
Hs.100000	S100 calcium binding protein A8 (calgranulin A)		Hs.370937	TAP binding protein (tapasin)
Hs.75367	Src-like-adaptor		Hs.232068	transcription factor 8 (represses interleukin 2 expression)
Hs.303157	T cell receptor beta locus		Hs.114360	transforming growth factor beta-stimulated protein TSC-22
Hs.256278	tumor necrosis factor receptor superfamily, member 1B		Hs.83429	tumor necrosis factor (ligand) superfamily, member 10
Hs.355307	tumor necrosis factor receptor superfamily, member 7		Hs.81791	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
			Hs.109225	vascular cell adhesion molecule 1

Figure 1.

Gene expression profiles generated from kidney biopsies according to different clinical classes. Classes represented were histologically confirmed and comprised healthy kidney donors (C), transplanted kidneys with stable function on full immunosuppression (TX), and kidneys undergoing acute rejection (AR). (A) Hierarchical clustering (unsupervised) of gene expression profiles from kidney biopsies. (B) Functional categories of genes up- or down-regulated from kidney biopsies. Functional gene categories were defined using gene names and annotations available from public domain databases. (C) Up-regulated immune/inflammation response genes identified in kidney biopsies from different clinical classes. The kidneys undergoing acute rejection were compared with stable immunosuppressed recipients (AR vs. TX); the 44 genes shown are up-regulated in AR (see Figure 2B). Stable

immunosuppressed recipients were compared with healthy donor kidney controls (TX vs. C); the 45 genes shown are up-regulated in TX (see Figure 2B).

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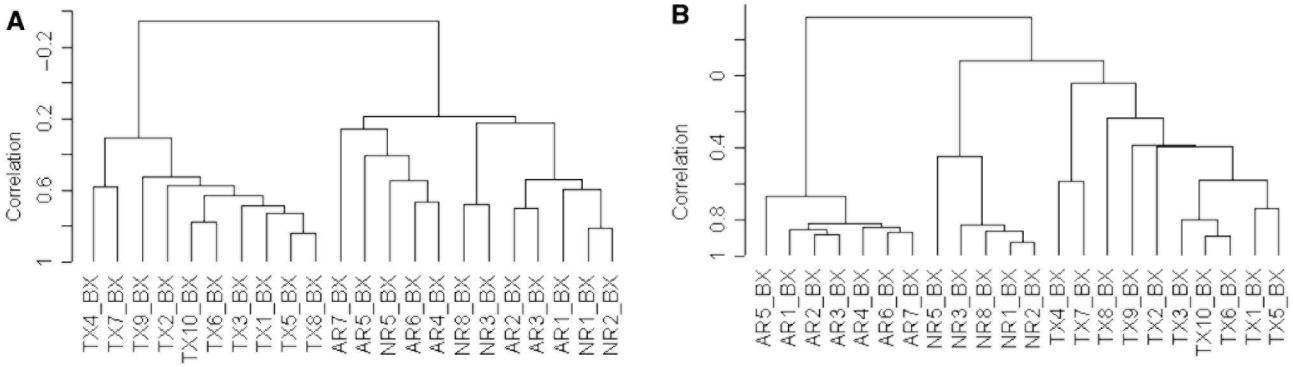

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C

Unigene ID	Gene	Function
Hs.155566	CASP2 and RIPK1 domain containing adaptor with death domain	Apoptosis
Hs.272499	dehydrogenase/reductase (SDR family) member 2	Carbohydrate Metabolism
Hs.10649	basement membrane-induced gene	Cell Adhesion
Hs.83173	cyclin D3	Cell Growth/Regulation
Hs.54089	BRCA1 associated RING domain 1	Cell Growth/Regulation
Hs.748	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	Cell Growth/Regulation
Hs.26047	trinucleotide repeat containing 4	Cell Growth/Regulation
Hs.37167	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)	Cell Growth/Regulation
Hs.81256	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	Cell Growth/Regulation
Hs.127826	erythropoietin receptor	Cell Growth/Regulation
Hs.45743	HTGadenosine A2b receptor	Cell Growth/Regulation
Hs.174050	endothelial differentiation-related factor 1	Cell Growth/Regulation
Hs.2561	nerve growth factor, beta polypeptide	Cell Growth/Regulation
Hs.77171	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	Cell Growth/Regulation
Hs.225951	polymerase (DNA directed) sigma	DNA Repair/Metabolism
Hs.75258	H2A histone family, member Y	DNA Repair/Metabolism
Hs.180570	cytochrome P450, subfamily IVF, polypeptide 12	Drug Elimination/Metabolism
Hs.166079	cytochrome P450, subfamily IIIA, polypeptide 5 pseudogene 2	Drug Elimination/Metabolsim
Hs.82542	acyloxyacyl hydrolase (neutrophil)	Fatty Acid / Lipid Metabolism
Hs.327	interleukin 10 receptor, alpha	Immune/Inflammation Response
Hs.75367	Src-like-adaptor	Immune/Inflammation Response
Hs.1765	lymphocyte-specific protein tyrosine kinase	Immune/Inflammation Response
Hs.73946	endothelial cell growth factor 1 (platelet-derived)	Immune/Inflammation Response
Hs.76364	allograft inflammatory factor 1	Immune/Inflammation Response
Hs.10306	natural killer cell group 7 sequence	Immune/Inflammation Response
Hs.110309	HLA-F, CDA12	Immune/Inflammation Response
Hs.83968	integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	Immune/Inflammation Response
Hs.173374	endothelial and smooth muscle cell-derived neuropilin-like protein	Immune/Inflammation Response
Hs.155597	D component of complement (adipsin)	Immune/Inflammation Response
Hs.73885	HLA-G histocompatibility antigen, class I, G	Immune/Inflammation Response
Hs.172674	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	Immune/Inflammation Response
Hs.21486	signal transducer and activator of transcription 1, 91kDa	Intracellular Signaling
Hs.103854	docking protein 1, 62kDa (downstream of tyrosine kinase 1)	Intracellular Signaling
Hs.23205	membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)	Intracellular Signaling
Hs.6150	Rho-specific guanine nucleotide exchange factor p114	Intracellular Signaling
Hs.246857	mitogen-activated protein kinase 9	Intracellular Signaling
Hs.103854	docking protein 1, 62kDa (downstream of tyrosine kinase 1)	Intracellular Signaling
Hs.184411	Serum Albumin Gene (ALB)	Miscellaneous
Hs.30909	limkain b1	Not Defined
Hs.247280	chromosome 20 open reading frame 18	Not Defined
Hs.179703	tripartite motif-containing 14	Not Defined
Hs.181326	myotubularin related protein 2	Not Defined
Hs.306327	Homo sapiens mRNA; cDNA DKFZp434A012 (from clone DKFZp434A012), mRNA sequence	Not Defined
Hs.22174	hypothetical gene CG018	Not Defined
Hs.4750	hypothetical protein DKFZp564K0822	Not Defined
Hs.57079	Homo sapiens cDNA FLJ13267 fis, clone OVARC1000964, mRNA sequence	Not Defined
Hs.75864	chromosome 5 open reading frame 8	Not Defined
Hs.433269	chromosome 14 open reading frame 11	Not Defined
Hs.372549	hypothetical protein MGC40413	Not Defined
	EST	Not Defined
Hs.62576	KIAA1240 protein	Not Defined
	Homo sapiens TSC2, NTHL1/NTH1 and SLC9A3R2/E3KARP genes	Not Defined
Hs.77770	adaptor-related protein complex 3, mu 2 subunit	Protein Synthesis/Metabolism
Hs.169895	ubiquitin-conjugating enzyme E2L 6	Protein Synthesis/Metabolism
Hs.17639	ubiquitin protein ligase	Protein Synthesis/Metabolism
Hs.195464	filamin A, alpha (actin binding protein 280)	Structural
Hs.2477	ARP1 actin-related protein 1 homolog B, centractin beta (yeast)	Structural
Hs.80988	collagen, type VI, alpha 3	Structural
Hs.118400	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	Structural
Hs.1815	myosin, light polypeptide 3, alkali; ventricular, skeletal, slow	Structural
Hs.75770	retinoblastoma 1 (including osteosarcoma)	Transcription Factors and Gene Expression Regulators
Hs.380935	heat shock transcription factor 1	Transcription Factors and Gene Expression Regulators
Hs.96028	forkhead box D1	Transcription Factors and Gene Expression Regulators
Hs.382008	TIF1beta zinc finger protein [Homo sapiens], mRNA sequence	Transcription Factors and Gene Expression Regulators
Hs.181163	high-mobility group nucleosomal binding domain 2	Transcription Factors and Gene Expression Regulators

Figure 2.

Unsupervised and supervised hierarchical clustering of gene expression profiles generated from kidney biopsies according to different clinical classes. Classes were histologically confirmed and comprised transplanted kidneys with stable renal function on full immunosuppression (TX), kidneys undergoing acute rejection (AR), and kidneys with acute dysfunction but where rejection was not found on histological examination (NR). (A) Unsupervised hierarchical clustering of gene expression profiles. (B) Supervised clustering was performed using the 65 genes identified by a class comparison analysis using BRB ArrayTools as distinguishing AR from NR. C. The common names, Unigene numbers and

functional categories of the 65 genes that distinguished the AR from the NR clinical classes by kidney biopsy profiles.

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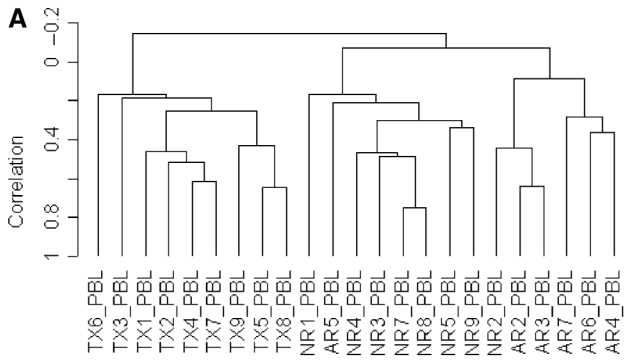

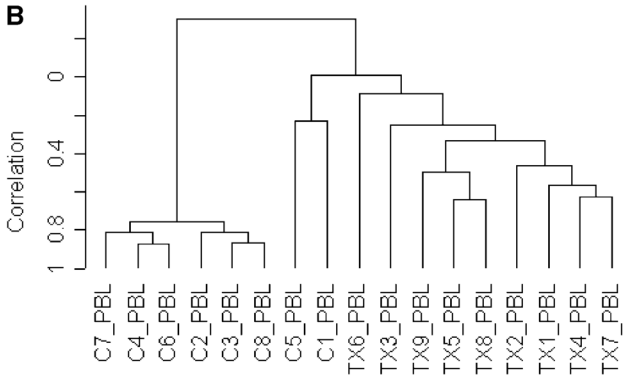

C

Functional Classes	AR vs. TX Up regulated	AR vs. TX Down regulated	Tx vs. C Up regulated	Tx vs. C Down regulated
Amino acid (and related products) metabolism	2	0	4	0
Apoptosis	2	0	3	2
Carbohydrate metabolism	0	1	1	0
Cell Adhesion	4	1	3	0
Cell growth/regulation	3	4	13	2
DNA Repair/metabolism	9	4	9	1
Drug elimination/metabolism	0	0	0	0
Energy (electron transport, ox phos and associated functions)	0	2	6	1
Extracellular matrix	0	0	0	0
Fatty acid / lipid metabolism	1	1	3	1
Glycosylation/glycosylase	1	1	3	0
Immune/inflammation response	2	6	13	3
Miscellaneous	3	1	5	3
Neuro related	0	11	0	0
Not defined	14	0	27	6
Oxidative stress	0	0	0	0
Protein synthesis metabolism	4	5	24	4
Signaling	8	3	12	8
Structural	3	0	5	3
Transcription Factors and gene expression regulators	8	8	17	4
Transport	1	1	3	1
Vesicle trafficking, endo, exocytosis	2	2	5	3
Total	**67**	**51**	**156**	**42**

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D

Function	AR vs. TX; up in AR		Tx vs. C; up in Tx	
	Unigene ID	Common Name	Unigene ID	Common Name
Immune/Inflammation	Hs.48516	beta-2-microglobulin	Hs.100194	arachidonate 5-lipoxygenase-activating protein
	Hs.406565	immunoglobulin kappa constant	Hs.75626	CD58 antigen, (lymphocyte function-associated antigen 3)
			Hs.15318	HS1 binding protein
			Hs.8024	IK cytokine, down-regulator of HLA II
			Hs.321045	IKK-related kinase epsilon; inducible IkappaB kinase
			Hs.84	interleukin 2 receptor, gamma (severe combined immunodeficiency)
			Hs.81337	lectin, galactoside-binding, soluble, 9 (galectin 9)
			Hs.78768	leukocyte receptor cluster (LRC) member 4
			Hs.380427	leukocyte specific transcript 1
			Hs.82116	myeloid differentiation primary response gene (88)
			Hs.2090	prostaglandin E receptor 2 (subtype EP2), 53kDa
			Hs.425339	stem cell growth factor; lymphocyte secreted C-type lectin
			Hs.432922	thioredoxin
Transcription Factors/Gene Expression Regulation	Hs.184298	cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)	Hs.25601	chromodomain helicase DNA binding protein 3
	Hs.75450	delta sleep inducing peptide, immunoreactor	Hs.7943	chromosome 19 open reading frame 2
	Hs.76507	LPS-induced TNF-alpha factor	Hs.285313	core promoter element binding protein
	Hs.348999	nuclear transcription factor Y, alpha	Hs.74578	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 9 (RNA helicase A, nuclear DNA helicase II; leukophysin)
	Hs.5409	RNA polymerase I subunit	Hs.347340	H-2K binding factor-2
	Hs.155321	serum response factor (c-fos serum response element-binding transcription factor)	Hs.1149	Human mRNA for pre-mRNA splicing factor SRp20, 5'UTR (sequence from the 5'cap to the start codon)
	Hs.184669	zinc finger protein 144 (Mel-18)	Hs.2780	jun D proto-oncogene
	Hs.156000	zinc finger protein 161 homolog (mouse)	Hs.920	modulator recognition factor 1
			Hs.287994	nuclear receptor co-repressor 2
			Hs.123654	PCF11p homolog
			Hs.183037	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
			Hs.18192	serine/arginine repetitive matrix 1
			Hs.80642	signal transducer and activator of transcription 4
			Hs.180677	splicing factor 1
			Hs.406186	splicing factor 3b, subunit 4, 49kDa
			Hs.155160	Splicing factor, arginine/serine-rich, 46kD
			Hs.155188	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa
Cell Growth/Regulation	Hs.77613	ataxia telangiectasia and Rad3 related	Hs.84264	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B
	Hs.106070	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	Hs.51692	BIA2
	Hs.159557	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Hs.108809	chaperonin containing TCP1, subunit 7 (eta)
			Hs.799	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)
			Hs.2707	G1 to S phase transition 1
			Hs.388623	LIM domain only 1 (rhombotin 1)
			Hs.78890	numb homolog (Drosophila)
			Hs.374491	proliferation-associated 2G4, 38kDa
			Hs.75551	Ras suppressor protein 1
			Hs.78944	regulator of G-protein signaling 2, 24kDa
			Hs.110457	Wolf-Hirschhorn syndrome candidate 1
			Hs.116237	vav 1 oncogene
			Hs.7165	zinc finger protein 259
DNA Metabolism	Hs.177766	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)	Hs.154868	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
	Hs.85004	centromere protein B, 80kDa	Hs.46423	H4 histone family, member G
	Hs.77254	chromobox homolog 1 (HP1 beta homolog Drosophila)	Hs.247817	histone family member
	Hs.28777	H2A histone family, member L	Hs.56	phosphoribosyl pyrophosphate synthetase 1
	Hs.795	H2A histone family, member O	Hs.76556	protein phosphatase 1, regulatory (inhibitor) subunit 15A
	Hs.795	H2A histone family, member O	Hs.180455	RAD23 homolog A (S. cerevisiae)
	Hs.155462	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	Hs.74592	special AT-rich sequence binding protein 1 (binds to nuclear matrix/scaffold-associating DNA's)
	Hs.79411	replication protein A2, 32kDa	Hs.332848	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
	Hs.84981	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kDa)	Hs.75248	topoisomerase (DNA) II beta 180kDa

Figure 3.

Gene expression profiles generated from peripheral blood lymphocytes (PBLs) according to different clinical classes. Classes comprised PBLs from patients with transplanted kidneys and stable renal function on full immunosuppression (TX), patients undergoing acute rejection (AR), patients with acute dysfunction but where rejection was not found on histological examination (NR), and PBLs from healthy blood donors (C). (A) Hierarchical clustering (unsupervised) of gene expression profiles from PBLs comparing the three transplant patient classes (TX, AR, NR). (B) Hierarchical clustering (unsupervised) of healthy donor PBLs (C) demonstrated distinct separation from the gene profiles generated from stable, well-functioning transplant recipient PBLs (TX). (C) Functional categories for the genes up or down-regulated according to different clinical classes. The PBLs from

patients undergoing acute rejection were compared with stable immunosuppressed recipients (AR vs. TX); stable immunosuppressed recipients were compared with healthy blood donor controls (TX vs. C). (D) Up-regulated genes identified in PBLs from different clinical classes. The PBLs from recipients undergoing acute rejection were compared with stable immunosuppressed recipients (AR vs. TX); and stable immunosuppressed recipients were compared with healthy blood donor controls (TX vs. C).

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Table 1

Clinical and demographic data of patients entered into the study

Patient ID	BX	PBL	Age	Sex	Immunosuppression	Histopathology	LD/CAD	Scr (mg/dL)	Days post TX
C1	•		38	Female				0.8	
C2	•		42	Male				0.9	
C3	•		35	Female				0.6	
C4	•		39	Female				0.9	
C5	•	•	39	Male				1.2	
C6	•		44	Male				0.8	
C7	•		36	Male				1.2	
C8	•		35	Female				0.8	
C9	•		50	Female				0.6	
AR1	•	•	42	Male	CsA/MMF/P	BanffIIA	CAD	12	285
AR2	•	•	28	Male	FK/MMF/P	BanffIIA	LD	5.9	1467
AR3	•	•	18	Male	CsA/MMF/P	BanffIA	CAD	2.2	119
AR4	•	•	28	Female	FK/MMF/P	BanffIIA	CAD	1.5	366
AR5	•	•	26	Female	CsA/MMF/P	Borderline	CAD	2	278
AR6	•	•	55	Male	SRL/MMF/P	Borderline	CAD	2.9	68
AR7	•	•	35	Male	SRL/MMF/P	BanffIA	CAD	2	184
TX1	•		51	Male	CsA/MMF/P	Normal	CAD	1.5	932
TX2	•		56	Male	CsA/MMF/P	Normal	LD	1.3	911
TX3	•		52	Male	CsA/MMF/P	Normal	CAD	1.2	902
TX4	•		31	Female	CsA/MMF/P	Normal	LD	1.1	651
TX5	•		53	Female	CsA/MMF/P	Normal	LD	1.1	689
TX6	•		32	Male	CsA/MMF/P	Normal	LD	1.6	776
TX7	•		46	Female	CsA/MMF/P	Normal	CAD	1.2	713
TX8	•		61	Male	CsA/MMF/P	Normal	CAD	0.9	733
TX9	•		44	Male	CsA/MMF/P	Normal	LD	1.8	718
TX10	•		21	Male	CsA/MMF/P	Normal	CAD	1.5	674
TXPBL1		•	38	Male	CsA/MMF/P		CAD	1.4	461
TXPBL2		•	57	Female	FK/MMF/P		LD	1.3	42
TXPBL3		•	65	Male	CsA/MMF/P		CAD	1.5	213

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Patient ID	BX	PBL	Age	Sex	Immunosuppression	Histopathology	LD/CAD	Scr (mg/dL)	Days post TX
TXPBL4		•	65	Female	FK/MMF/P		CAD	0.8	246
TXPBL5		•	36	Female	CsA/MMF/P		CAD	1.1	1278
TXPBL6		•	68	Male	CsA/MMF/		CAD	1.7	376
TXPBL7		•	39	Male	SRL/MMF/P		CAD	0.9	36
TXPBL8		•	61	Female	CsA/MMF/P		CAD	0.9	1491
TXPBL9		•	46	Male	SRL/MMF/P		LD	1.2	81
NR1	•	•	55	Male	CsA/MMF/P	CNI toxicity	LD	1.7	456
NR2	•	•	38	Male	FK/MMF/P	CNI toxicity	LD	2.3	155
NR3	•	•	61	Male	SRL/MMF/P	ATN	LD	5.2	11
NR4	•	•	43	Male	CsA/MMF/P	CNI toxicity	CAD	3.8	262
NR5	•	•	35	Male	CsA/MMF/P	ATN	CAD	6.3	16
NR6		•	35	Female	FK/MMF/P	CNI toxicity	CAD	2.6	37
NR7		•	44	Male	SRL/MMF/P	ATN	CAD	6.3	40
NR8	•	•	22	Female	FK/MMF/P	FSGS	LD	3.3	78
NR9		•	58	Male	CsA/MMF/P	ATN	CAD	5	47

BX, biopsy; PBL, peripheral blood lymphocytes; CsA, cyclosporine; MMF, mycophenolate mofetil; P, prednisone; FK, tacrolimus; SRL, sirolimus; CAD, cadaveric; LD, live donor; Scr, serum creatinine; ATN, actute tubular necrosis; CNI, calcineurin inhibitor; FSGS, focal segmental glomerulosclerosis.

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Table 2

Class prediction analysis of kidney biopsy and PBL samples using multiple statistical alogorithms

Classifier	Sample type	Phenotype	Number of samples	Compound covariate predictor	Linear discriminant analysis	1-nearest neighbor	3-nearest neighbor	Nearest centroid	Support vecor machines	Classifier p-value	Average number of genes in classifier
Tx vs. AR	Biopsy	Tx	10	100%	100%	100%	100%	94%	100%	0.005–<0.0005	2837
		AR	7								
	PBL	Tx	9	93%	93%	93%	93%	93%	93%	0.009–0.005	743
		AR	6								
AR vs. NR	Biopsy	AR	7	83%	75%	67%	92%	83%	67%	0.247–0.017	73
		NR	5								
	PBL	AR	6	79%	79%	79%	79%	79%	79%	0.121–0.083	77
		NR	8								
Tx vs. NR	Biopsy	Tx	10	100%	100%	100%	100%	100%	100%	0.002–<0.0005	3792
		NR	5								
	PBL	Tx	9	94%	94%	94%	94%	94%	94%	0.003–<0.0005	1812
		NR	8								
Tx vs. AR/NR	Biopsy	Tx	10	100%	100%	100%	100%	100%	100%	<0.0005	3728
		AR/NR	12								
	PBL	Tx	9	100%	100%	100%	100%	100%	100%	<0.0005	1017
		AR/NR	14								
C vs. Tx	Biopsy	C	9	100%	100%	100%	100%	100%	100%	<0.0005	5165
		Tx	10								
	PBL	C	8	88%	88%	88%	88%	88%	94%	0.02–0.001	1910
		Tx	9								

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Table 3

Real time PCR analysis of selected gene transcripts

Unigene ID	Gene name	Gene list	Upregulated chip in	Fold difference by chip	Upregulated Q-PCR in	Fold difference by Q-PCR	Agreement
Hs. 73946	Enothelial cell growth factor (platelet-derived)	TX vs. AR	AR	2.6	AR	218	Yes
Hs. 155597	D component of complement (adipsin)	TX vs. AR	AR	1.9	AR	982	Yes
Hs. 76364	Allograft inflammatory factor 1	TX vs. AR	AR	1.6	AR	529	Yes
Hs.73885	HLA-G histocompatibility antigen, class I, G	C vs. TX	TX	3.2	TX	$>10^5$	Yes
Hs. 6150	Rho-specific guanine nucleotide exchange factor p114	C vs. TX	TX	2.5	C	7	No
Hs. 79356	Lysosomal-associated multispanning membrane protein-5	TX vs. AR	AR	2.7	AR	6	Yes
Hs. 21486	Signal transducer and activator of transcription 1, 91 kDa	AR vs. NR	AR	3.98	AR	6105	Yes
		TX vs. AR	AR	3.99	AR	6	Yes
Hs. 327	Interleukin 10 receptor, alpha	AR vs. NR	AR	2.16	AR	121	Yes
		TX vs. AR	AR	1.92	AR	266	Yes
Hs. 202	Benzodiazapine receptor (peripheral)	AR vs. NR	AR	2.14	AR	4	Yes
Hs. 75367	Src-like-adaptor	TX vs. AR	AR	3.65	AR	72	Yes
Hs. 425777	Ubiquitin-conjugating enzyme E2L 6	AR vs. NR	AR	2.47	AR	2	Yes
Hs. 193852	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	AR vs. NR	NR	2.26	NR	72	Yes
		TX vs. AR	TX	3.66	TX	4	Yes
Hs. 83968	Integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	AR vs. NR	AR	2.27	AR	161	Yes
		TX vs. AR	AR	2.41	AR	7	Yes
Hs. 184411	Human serum albumin (ALB) gene	AR vs. NR	NR	3.83	NR	143	Yes
		TX vs. AR	TX	4.77	TX	7	Yes
Hs. 1765	Lymphocyte-specific protein tyrosine kinase	AR vs. NR	AR	1.97	AR	36	Yes
		TX vs. AR	AR	1.66	AR	2	Yes

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