

Clinical Implications and Longitudinal Alteration of Peripheral Blood Transcriptional Signals Indicative of Future Cardiac Allograft Rejection

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Background: We have previously demonstrated that a peripheral blood transcriptional profile using 11 distinct genes predicts onset of cardiac allograft rejection weeks to months prior to the actual event.

Methods: In this analysis, we ascertained the performance of this transcriptional algorithm in a Bayesian representative population: 28 cardiac transplant recipients who progressed to moderate to severe rejection; 53 who progressed to mild rejection; and 46 who remained rejection-free. Furthermore, we characterized longitudinal alterations in the transcriptional gene expression profile before, during and after recovery from rejection.

Results: In this patient cohort, we found that a gene expression score (range 0 to 40) of ≤ 20 represents very low risk of rejection in the subsequent 12 weeks: 0 progressed to treatable (ISHLT Grade $\geq 3A$) rejection; 16 of 53 (30%) from the intermediate group (those who progressed to ISHLT Grade 1B or 2) and 13 of 46 (28%) controls (who remained Grade 0 or 1A) had scores ≤ 20 . A gene score of ≥ 30 was associated with progression to moderate to severe rejection in 58% of cases. These two extreme scores (≤ 20 or ≥ 30) represented 44% of the cardiac transplant population within 6 months post-transplant. In addition, longitudinal gene expression analysis demonstrated that baseline scores were significantly higher for those who went on to reject, remained high during an episode of rejection, and dropped post-treatment for rejection ($p < 0.01$).

Conclusions: The use of gene expression profiling early after transplantation allows for separation into low-, intermediate- or high-risk categories for future rejection, permitting development of discrete surveillance strategies. *J Heart Lung Transplant* 2008;27:297-301. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

The key challenge in managing cardiac transplant patients continues to be developing refined methods for

balancing the risk of rejection with immunosuppressant drug toxicity.^{1,2} In this quest, advances in surveillance techniques for cardiac allograft rejection have led to the emergence of non-invasive transcriptional profiling in peripheral blood. As a part of the Cardiac Allograft Rejection Gene Expression Observational (CARGO) study, the development and validation of a gene expression signature was described consisting of 11 informative genes (and 9 normalization and control genes) whose expression in peripheral blood mononuclear cells correlated with the absence or presence of endomyocardial biopsy-proven moderate to severe cardiac allograft rejection.³ Subsequent work in this study has elaborated the post-transplant time-dependent clinical use of this gene expression signature⁴ and even identified a potential link with the development of chronic coronary artery vasculopathy.⁵

The CARGO study work was recently extended by the demonstration that altered transcriptional signals of the 11 informative genes were evident early during clinical quiescence in patients when assessed weeks to

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Table 1. List of Genes Significantly Associated With Future Acute Cellular Rejection^a

Expression	Gene	Description	
Decreased	IL1R2	Interleukin-1 receptor, type II	
	FLT3	fms-related tyrosine kinase 3	
	ITGAM	Integrin alpha M	
	IL1R1	Interleukin-1 receptor, type I	
	TSC22D3	TSC22 domain family 3	
	FKBP5	FK 506 binding protein 5	
	THBS1	Thrombospondin 1	
	CD163	CD163 antigen	
	Increased	PDCD1	Programmed cell death 1
		ADA	Adenosine deaminase
GMZA		Granzyme A	
TRBC1		T-cell receptor beta constant 1	
FLT3LG		fms-related tyrosine kinase 3 ligand	
NFKB1		Nuclear factor of kappa light-chain enhancer in B-cells 1	
TNF		Tumor necrosis factor	

Informative genes from the gene expression signature are represent in boldface. Three steroid-responsive genes from the gene expression signature (IL1R2, FLT3 and ITGAM) were decreased in patients who went onto reject, as were an additional five functionally steroid-related genes. One T-cell activation gene from the gene expression signature (PDCD1) was significantly increased in patients who progressed to rejection, as were an additional six functionally related T-cell genes.

^aRefer to Mehra et al.⁶

months before histologic manifestation of rejection.⁶ Thus, the investigation provided an indication for a predictive signal for transcriptional profiling. The components of the signature responsible for this association were primarily genes involved in steroid responsiveness and T-cell activation (Table 1), and this was further substantiated through the detection of similar associations in a number of other functionally related genes.⁶ As noted in the Discussion section, although the molecular association is characterized, the limitations of a case-control study design restrict the clinical and causal conclusions one can make. In clinical practice, patients are encountered in an unselected and random manner without the privilege of weeding-out those who fall into the “gray zone.” In the context of cardiac allograft rejection, these are patients with endomyocardial biopsy histologic grades of rejection interpreted as “intermediate” or mild and are usually followed clinically without a clear treatment imperative.

This follow-on analysis from the CARGO study was designed to support two objectives. First, we sought to ascertain how the peripheral blood transcriptional profiling signature might perform in the setting of a more representative patient population—which includes patients who progress to all grades of histologic rejection adjusted by using Bayesian analysis—for prevalence as would be encountered in routine clinical practice. The

second objective was to characterize longitudinal serial alterations in the transcriptional gene expression profile at 3 distinct time-points: weeks to months before rejection, during rejection, and after recovery from cardiac rejection.

METHODS

Patients

All patient samples, including those from the original case-control study, were obtained as a part of the CARGO study. The CARGO study enrolled 629 subjects between September 2001 and June 2003 in protocols approved by the institutional review boards at each of the participating centers. Principal inclusion criteria for this analysis were: (a) absence of clinical signs and symptoms; (b) current ISHLT Grade 0 or 1A by endomyocardial biopsy; (c) absence of cardiac dysfunction as measured by invasive hemodynamics or echocardiogram; and (d) absence of International Society for Heart and Lung Transplantation (ISHLT) Grade $\geq 3A$ rejection, graft dysfunction or administration of rejection therapy within the previous 30 days. All patients in the CARGO cohort who developed ISHLT Grade $\geq 3A$ rejection within 12 weeks were identified, and comprised the rejection group. The control group was comprised of patients who satisfied the inclusion criteria, remained rejection-free during the same time period, and were matched with the rejection group by demographic characteristics, time since transplant and immunosuppression.

Because intermediate biopsy outcomes were not included in the previously described case-control study, additional patients who met the inclusion criteria, were ≤ 180 days post-transplant, and developed ISHLT Grade 1B or 2 mild rejections within 12 weeks were randomly selected from the CARGO cohort to further evaluate the gene expression test. Combining this intermediate group with the case-control groups, we then estimated expected outcomes in a representative clinical population using a Bayesian analysis.

Longitudinal gene expression profiles were measured for the rejection and control patients at 3 distinct time-points. These comprised the index (pre-rejection), rejection event (or time-matched control) and the post-rejection (or time matched control) samples, respectively. These sample triplets from study patients were used to detect different longitudinal patterns between the rejection and control groups. Because the primary objective of the CARGO study was to study discriminatory interactions between extremes of histologic findings (no clinical rejection and treatable rejection), not enough longitudinal profiles for the intermediate group were available to analyze and report.

Gene Expression Profiling

Gene expression analysis was performed using AlloMap molecular expression testing (XDx, Inc., South San Francisco, CA). Peripheral blood mononuclear cells were isolated from 8 ml of venous blood using density gradient centrifugation (CPT; Becton-Dickinson, Franklin Lakes, NJ), and samples were frozen in lysis buffer (RLT; Qiagen, Valencia, CA) within 2 hours of phlebotomy. Total RNA was isolated from each sample (RNeasy, Qiagen). For each of the 20 genes in the gene expression test (11 informative, 6 normalization, 3 control), triplicate 10- μ l quantitative real-time polymerase chain reaction (PCR) reactions were performed on an ABI 7900HT system with FAM-TAMRA probes and standard TaqMan protocols (Applied Biosystems, Foster City, CA) on cDNA from 0.5 ng of total RNA. Gene expression values, measured by threshold cycle (C_T), were used to calculate the gene expression score, which ranges from 0 to 40.³

Statistical Analysis

A receiver operator characteristic (ROC) curve was constructed to derive threshold scores for further analysis. The qualifying (satisfying all study inclusion criteria) stable patients either: progress to ISHLT Grade $\geq 3A$ rejection within 12 weeks (rejection group); progress to ISHLT Grade 1B or 2 (intermediate group); or remain stable with ISHLT Grade 0 or 1A (control group). To extrapolate from the case-control study (i.e., adjust for spectrum bias), the gene expression profile results were generated for a representative set of intermediate-group patients. The performance of each group was then weighted according to the distribution of patient samples in the clinical population, calculated from the CARGO data.³

Longitudinal changes in gene expression score in the rejection patients from the index sample to rejection and post-rejection samples were compared with the corresponding control samples by using repeated-measures analysis of variance (ANOVA).

RESULTS

Score Thresholds and Outcomes

Figure 1 shows the ROC curve constructed from the original 74 patients in the case-control study⁶ with encounters ≤ 180 days post-transplant. No patients with gene expression scores of ≤ 20 had rejection episodes in the next 12 weeks ($n = 19$), whereas 58% of patients with gene expression scores of ≥ 30 did have rejection episodes.

Estimates for Clinical Populations

Clinically stable patients who satisfied the inclusion criteria of this study fell into one of three groups: the

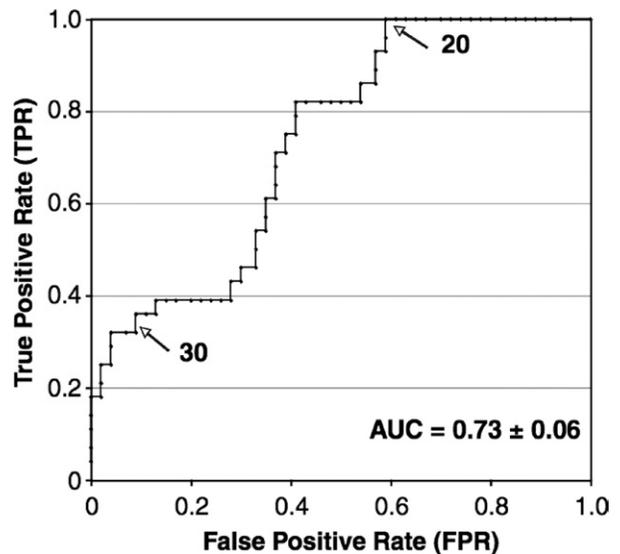


Figure 1. Receiver operator characteristic (ROC) curve for association of gene expression score with future acute cellular rejection at ≤ 180 days post-transplant ($n = 74$). The area under the curve (AUC) is 0.73 ± 0.06 ($p = 0.02$). The true-positive rate, or sensitivity, is the fraction of index samples in the rejection group that score above a gene expression score cut-off. The false-positive rate, or specificity, is the fraction of index samples in the control group that score above the same cut-off.

rejection or case group (who progressed to ISHLT Grade $\geq 3A$); an intermediate group (who progressed to ISHLT Grade 1B or 2); or the control group (remained ISHLT Grade 0 or 1A). For this analysis, gene expression profiles were used from 28 rejection, 53 intermediate and 46 control patients at ≤ 180 days post-transplant, respectively.

In the CARGO database, containing data from 629 cardiac allograft patients monitored at 8 transplant centers, there were 382 qualifying samples at ≤ 180 days post-transplant that could be assigned to each of these three groups: 5.8% progressed to ISHLT Grade $\geq 3A$; 13.1% progressed to ISHLT Grade 1B or 2; and 81.0% remained ISHLT Grade 0 or 1A. None of the 28 (0%) case patients, 16 of 53 (30%) of the intermediate patients and 13 of the 46 (28%) control patients had gene expression test scores of ≤ 20 . The estimate of the expected fraction of patients to have gene expression test scores of ≤ 20 in a clinical population (like the CARGO population) was then calculated as: $(5.8\% * 0\%) + (13.1\% * 30\%) + (81.0\% * 28\%) = 26.6\%$. The estimate of the expected fraction of patients to have gene expression test scores ≥ 30 in a clinical population (like the CARGO population) was calculated as: $(5.8\% * 39\%) + (13.1\% * 23\%) + (81.0\% * 15\%) = 17.4\%$.

Longitudinal Patterns

Figure 2 depicts serial scores at 3 time-points (index, rejection/no-rejection and post-rejection/no-rejection).

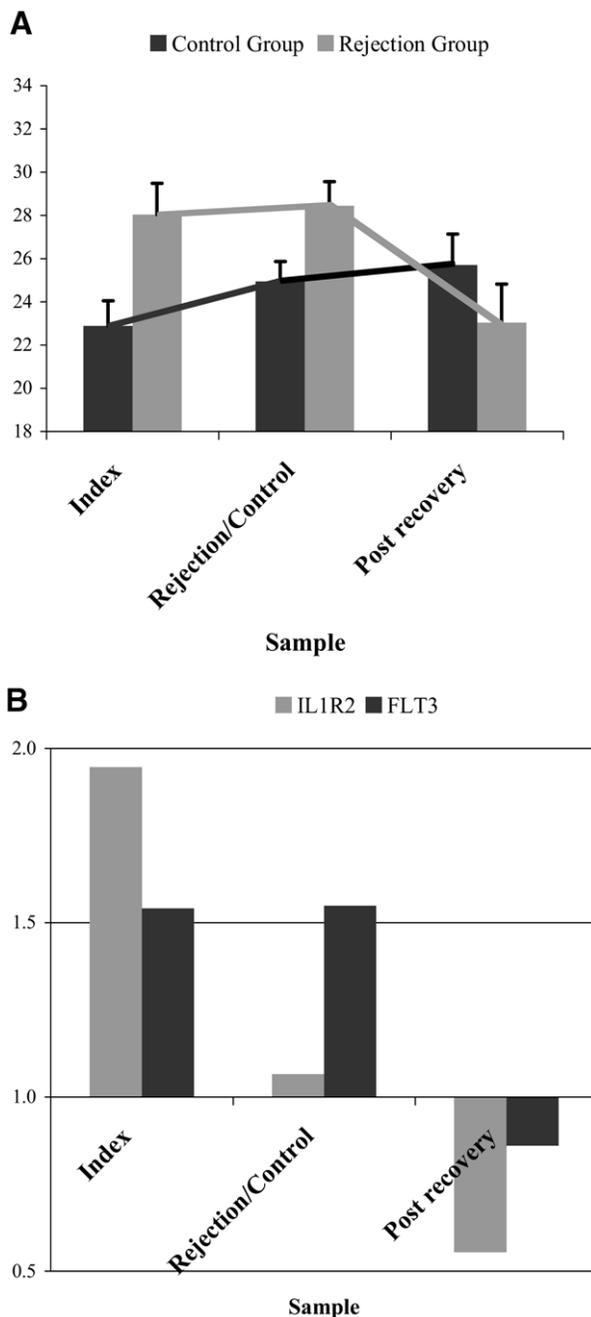


Figure 2. Longitudinal comparison of gene expression scores and steroid-responsive gene expression at three time-points: (1) index (pre-rejection); (2) acute rejection or control; and (3) post-rejection recovery/control samples. (Analyses were performed using repeated-measures ANOVA.) The mean gene expression scores and relative expression values for IL1R2 and FLT3 are shown for patients with index samples at ≤ 180 days post-transplant. (A) Serial gene expression score analysis. The score is significantly higher in index and rejection samples for the rejection group vs the control group and drops significantly after administration of rejection therapy, as indicated in the post-recovery samples. In the control group, which did not receive rejection therapy, this effect is not seen (repeated-measures ANOVA, $p = 0.0002$). (B) Serial analysis of IL1R2 and FLT3 relative gene expression measured as fold difference (calculated as $2^{(\text{mean rejection group } C_T - \text{mean control group } C_T)}$). From the index

Index samples were collected 103 ± 51 days post-transplant for the case group and 96 ± 46 days post-transplant for the control group. Rejection samples were collected at 139 ± 60 days post-transplant compared with 131 ± 54 days for the control group. Post-recovery samples were collected 155 ± 61 days post-transplant compared with 170 ± 71 for the control group. None of these differences were significant.

Case (rejection) patients demonstrated higher gene expression scores at the index sample and during rejection compared with the control group ($p = 0.0002$). Gene expression scores in rejectors showed an increase from the index sample to rejection (7.2%) and a subsequent decrease (−18%) in gene expression after augmentation of immunosuppression. The control group demonstrated a slight increase in gene expression scores with time (Figure 2). The expression of IL1R2 increased 2-fold from the index sample to the post-recovery sample for patients in the rejection group, whereas its expression decreased in the control group ($p = 0.0001$). The expression of FLT3 followed a similar pattern and increased by 58% from index sample to post-recovery sample for patients treated with rejection therapy, whereas expression decreased for the control group by 26% ($p = 0.03$; Figure 2).

DISCUSSION

The results of our investigation, designed to understand the potential implications of the predictive nature of peripheral blood transcriptional signals in a prevalent cardiac allograft population, suggest that 44% of the early post-transplant population (in the first 6 months) will have a gene expression score that provides discriminatory ability of either allocating patients into a low- or high-risk group for future rejection. Thus, a gene expression score of ≤ 20 is associated with very low risk of rejection in the subsequent 12 weeks. In such patients, none (0%) progress to moderate to severe (Grade $\geq 3A$) rejection, whereas 30% of these patients will have follow-up histologic data that fall within the intermediate group (Grade 1B or 2), with unclear implications for treatment. At the other extreme is a gene score of ≥ 30 , which is associated with a 58% incidence of progression to moderate to severe ($\geq 3A$) rejection. According to our Bayesian estimates, in aggregate, these two extreme scores (≤ 20 or ≥ 30) are prevalent in 44% of the cardiac transplant population within 1 to 6 months post-transplant. In the low-risk group of patients, one could develop and validate strategies that include less frequent endomyocardial biopsies or more

sample to the post-recovery sample there is a significant increase in expression in the rejection group. In the control group, which did not receive rejection therapy, this effect is not seen ($p = 0.0001$).

aggressive steroid weaning. Conversely, a higher score points to the need for closer clinical surveillance and caution in aggressive algorithmically ascertained steroid weaning.

Longitudinal gene expression analysis demonstrates that the scores are significantly higher for those who go on to reject at baseline, increase modestly during an episode of rejection, and drop lower after treatment for rejection. As would be expected, the control group (non-rejectors) patients demonstrated a gradual increase in gene expression scores at the three follow-up time-points. This finding is commensurate with ongoing corticosteroid weaning, which alters the gene expression score via modulation of the three steroid-responsive genes (IL1R2, ITGAM and FLT3) as noted in the discovery phase of this investigation.⁶

Importantly, there are qualitative aspects of gene expression that demonstrate shifts at the various time-points studied. The predictive power of the gene expression algorithm is driven predominantly by the altered expression levels of corticosteroid-responsive genes, IL1R2 and FLT3, involved in bone marrow mobilization of hematopoietic precursors, and PDCD1, a gene involved in T-cell activation pathways.⁶ These genes are predominantly responsible for the elevated gene expression score in the predictive phase (weeks to months *before* rejection onset). However, during acute rejection, the significant increase in gene expression score represents increasing contributions from those in the corticosteroid-responsive gene set (FLT3 and IL1R2), but now also includes those genes reflecting the erythroid lineage (WDR40A and MIR), which may denote additional deployment of an erythroid precursor mobilization stress response.⁷ These changes may represent a reduced response to glucocorticoids and bone marrow mobilization during rejection.

The limitations listed in the Discussion section of our previous report⁶ largely apply to this extension to that work as well, particularly the difficulty inherent in generalizing from a case-control study to a clinical population. Given the low incidence of moderate/severe rejection, a randomized study design would require a much larger and costly study population. Furthermore, we have been unable to robustly define the longitudinal gene expression profile within the subset of intermediate patient groups (those that progress to mild forms of histologic rejection). The clinical significance of this category of rejection remains uncertain and has been downgraded by the new rejection classification system developed by the International Society of Heart and Lung Transplantation.⁸

Recently, a debate has ensued about the most appropriate way of ascertaining significance in the context of gene expression and gene association studies, particularly those that handle genome-wide associations with an astonishing number of genes.⁹ Those investigators and others¹⁰ have

concluded that the best validation of gene expression studies is less in the statistical handling of such data, but more importantly resides in the ability to consistently reproduce the estimates of association in independent data sets representative of the same disease-state phenotype. Thus, although our exploratory and clinical implication analyses are steps in the right direction, only real-world replication will finally establish the validity of these findings. Ideally, we call for validation within the context of randomized intervention trials that seek to study conventional surveillance vs gene expression profiling-based patient management in the early time period after transplantation.

In conclusion, we believe this work extends the results of the previous exploratory study⁶ as it has offered potential estimates of performance of gene expression profiling within a contemporary cardiac transplantation patient population by providing the ability to separate patients into low-, intermediate- or high-risk categories for future rejection. Furthermore, we have also provided insight into longitudinal gene expression profiles in cardiac transplant recipients early after heart transplantation. These findings will form the foundation for the development of clinical strategies for individualization of immunosuppression and surveillance for rejection after cardiac transplantation.

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