Gene-Based Bio-Signature Patterns and Cardiac Allograft Rejection

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KEYWORDS

• Gene expression • Transplantation • Rejection
• Transcriptional signaling • Genome

Successful cardiac transplantation requires surgical success in donor and recipient matching, hemodynamic restoration, and maintenance of immunologic stability. Although a clinical reality, cardiac transplantation represents a scarce resource with a restricted donor supply. Therefore, the notion of patient-centered multidisciplinary care to preserve and nourish the outcomes of these patients has emerged.[1] The surveillance for cardiac allograft rejection using an invasive endomyocardial biopsy has become entrenched in patient care algorithms.

Despite invasiveness and morbidity with this approach, most transplant centers use frequent cardiac biopsies to monitor patients, especially in the first year after transplantation. Clinicians feel an inherent comfort in the perceptive definitive nature of observing histologic changes in the allograft using a biopsy approach, and do so despite the various limitations of this histologic assessment.[2] This discussion focuses on describing the need for alternative approaches to allograft rejection monitoring, outlines the emerging opportunity of personalized medicine using gene-based biomonitoring, and argues for the advantages of using a peripheral gene-based bio-signature for optimal immunosuppression and improved outcomes.

USE AND ABUSE OF ENDOMYOCARDIAL BIOPSY

Oddly, the widespread adoption of histologic endomyocardial biopsy assessment has occurred without conclusive evidence for the benefit of current algorithmic approaches. In the first year, the cardiac allograft experiences a dynamic immunologic environment characterized by an initial period of heightened rejection risk in the first 3 months, continued but lessened rejection proclivity in the subsequent 3 months, and then a lull in rejection risk that is typically disturbed by altered immunosuppression exposure (changes in dose or metabolism) or activation of the immune system (eg, from a viral infection, pregnancy, blood transfusion, or other antigenic exposure, such as a vaccination) (**Fig. 1**).

In some patients, the allograft remains so immunologically mismatched that the rejection risk is never abrogated and close vigilance is needed. These individuals are characterized by frequent early rejections and inability to successfully wean corticosteroids. Thus, algorithms that use more frequent cardiac biopsies in the first 3 to 6 months, followed by increasing intervals thereafter, are typically noted in most surveillance programs. An individual is typically exposed to 13 to 15 cardiac

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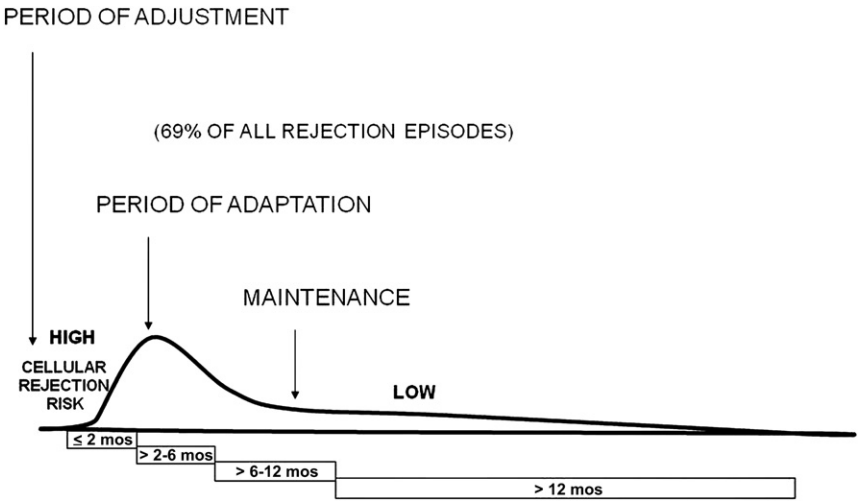

Fig. 1. Time-dependent dynamic rejection risk. The cluster effect of allograft rejection.

biopsy procedures in the first year after transplantation.[2]

The frequencies with which cardiac biopsies are performed have never been scientifically authenticated as sound but are anecdotally presumed to improve outcomes. This premise is based on the assumption that allograft infiltration by activated immune cells can be noted histologically before the severity of the rejection process advances to manifest hemodynamic compromise. This early recognition may allow for immune interruption through immunosuppression augmentation, although this remains unproven in the clinical armamentarium.

Interpretation of endomyocardial biopsy is fraught with inconsistency, and the grading system for histologic rejection is not necessarily linear in its severity scale, as molecular studies have now suggested.[3,4] A negative histology does little to provide comfort for near-term outcomes from rejection. Thus, a negative biopsy does not have any future rejection predictive capability and does not allow any mechanism for personalizing the frequency of monitoring between immunoprivileged and immunocompromised patient subsets.

This problem leads to unnecessary biopsies, with most showing no significant rejection. Although the first-year rejection rate described in per-patient rates (any of 13–15 first-year biopsies with significant rejection) continues to be approximately 30%, the per-biopsy rate of rejection discovery ranges from 2% to 3%. Therefore, these biopsies represent tests with high negative predictive values but poor positive predictive values. Clearly, an improvement in this monitoring system is needed, and a monitoring technology is needed that not only is noninvasive but also informs on future rejection proclivity through segregating patients into immune risk categories and identifying molecular patterns weeks to months before histologic damage. In this regard, the advent of gene-based peripheral blood monitoring has gained traction.

SINGLE GENES AND CARDIAC TRANSPLANT OUTCOME

Genetic mutations or single nucleotide polymorphisms may alter protein physiology and change functional responses to the alloimmune interaction. In other cases, they may influence allograft outcomes through altering pharmacology of immunosuppressive medications or influencing other intersecting organ systems. Various genetic polymorphisms in donor organs and recipients have been correlated with cardiac transplant outcomes. These polymorphisms are clustered in genes whose products are involved in alloimmune interactions (cytokines, chemokines, cell adhesion molecules), the renin–angiotensin–aldosterone system. or the transforming growth factor-β superfamily. Other applications of genetic polymorphism studies include the prediction of non–allograft-related outcomes (eg, the proclivity toward development of renal failure after transplant and pharmacogenetic interactions, which influence the metabolism of immunosuppressive drugs and thereby affect allograft outcomes). The authors' group provides a detailed discussion of these various genes in a previous review.[5]

The clinical usefulness of single genes is limited because these studies are observational, include small patient numbers, and use end points with

nonstandardized definitions and thresholds of abnormality (eg, cardiac allograft vasculopathy, chronic rejection, renal dysfunction). Furthermore, the implications of single-center findings from restricted patient cohorts become uncertain in applicability to ethnically diverse populations, for instance in African American heart transplantation.[6]

MULTI-GENE TRANSCRIPTOMIC SIGNALING FOR CURRENT REJECTION

The transcriptome, represented by mRNA, offers promise to simultaneously examine quantitative levels of various alloimmune and nonalloimmune gene expression during differing ambient states. The milieu of alloimmune interaction is a complex one, with gene expression in various domains of immune activation, cardiac injury, cardiac repair, and immune quiescence. Thus, it involves the allograft, peripheral blood elements, and likely organs such as the bone marrow and immune-active organs such as the thymus, lymphatic tissue, and spleen. Thus, it makes intuitive sense to examine the interaction of multiple genes and their relative expressions. Most of these studies have involved measurements of gene expression using polymerase chain reaction (PCR) from biopsy samples taken from the graft, or, in a few cases, from the peripheral blood, which is a more preferred approach.

Single-center Studies

Using quantitative PCR methods on 39 cytokine and related genes, Schoels and colleagues[7] examined gene expression in peripheral blood mononuclear cells from 44 patients who underwent cardiac allograft, classified according to the International Society for Heart and Lung Transplantation biopsy grades. Eight genes were significantly different between the grade 0 and 1 (none to mild rejection) groups compared with those who had grade 2 (significant rejection) or higher biopsies: *PRF1*, *GZMB*, *FAS*, *CCL5*, *CXCR3*, *CXCL5*, *PTGS2*, and *TGFB1*. Discriminant analysis on this training set was used to select five genes that, with a threshold determined post hoc, showed 82% agreement with biopsy samples of grades higher than 2, and 84% agreement with grades 0 and 1. No independent test set was used, however, to verify these intriguing results.

Horwitz and colleagues[8] performed a case-control study of 7 patients within a cohort of 189 who underwent cardiac transplantation. These investigators identified 91 gene transcripts differentially expressed in rejection compared with controls (false discovery rate <0.10). In samples taken from the same patients after rejection, 98% of transcripts returned toward control levels, displaying an intermediate expression profile for patients who underwent treatment for rejection ($P<.0001$). Quantitative real-time PCR supported significant differential expression for only the predictive markers CFLAR and superoxide dismutase 2 among 20 different expression markers investigated.

Multicenter Studies

The largest gene expression profiling study was the Cardiac Allograft Rejection Gene Expression Observation (CARGO) study. Using a multicenter prospective study design, this study group developed a multigene panel to distinguish clinically significant rejection from clinical quiescence.[9] More than 650 patients at eight centers were followed up, and peripheral blood samples and biopsy results from more than 7000 samples were accumulated and studied in pairs. The discovery phase included microarray analysis and knowledge-based approaches of peripheral blood mononuclear cell samples.

This phase was followed by the development of a quantitative real-time PCR assay on more than 250 genes from more than 250 unique patients. Use of these data, in combination with a centralized pathology panel to improve biopsy reading consistency, and several bio-informatic approaches led to the development of a linear 20-gene panel algorithm.

The CARGO gene expression panel, developed using linear discriminant analysis, is based on quantification of the abundance of peripheral blood mononuclear cell RNA using real-time PCR and is composed of a 20-gene classifier (11 informative and 9 housekeeping or control genes) that significantly discriminates absence of rejection from presence of moderate to severe histologic rejection. The genes involved represent several biologic pathways, including T-cell activation (*PDCD1*), T-cell migration (*ITGA4*), and mobilization of hematopoietic precursors (*WDR40A* and microRNA gene family cMIR), and steroid-responsive genes such as *IL1R2*, the decoy receptor for interleukin (IL)-2.

Rejection can be distinguished from quiescence through determining a relative gene expression score on a scale of 0 to 40, wherein higher scores indicate increased risk for rejection. The performance of the algorithm, validated in an independent test set more than 2 months after transplantation, denotes a negative predictive value higher than 99.5% at preselected score thresholds, but a low positive predictive value.[10]

TRANSCRIPTIONAL SIGNALING FOR PREDICTING ALLOGRAFT REJECTION RISK

A clinical advance allowing prediction of future risk of allograft rejection and compartmentalization of risk categories for individual patients would be desirable. This need is greatest during the early months after heart transplantation when rejection incidence is greatest.

The CARGO group recently investigated the usefulness of this multigene panel initially developed for ongoing rejection as a test that may predate the occurrence of rejection by weeks to months.[11] The principle design was to investigate 104 cardiac allograft recipients using a case-control study design to distinguish transcriptional profiles of blood samples obtained during histologic quiescence within 12 weeks of a future rejection episode, compared with persistent quiescence on similar follow-up. The 11 informative gene panel, commercially called Allomap, found component genes that possessed qualitative and quantitative predictive capacity. Only *IL1R2* was significantly decreased with future rejection, and three other genes (*FLT3*, *ITGAM*, and *PDCD1*) showed borderline significant changes between the groups.

Gene expression measurements of additional genes in pathways associated with *PDCD1*, *IL1R2*, and *FLT3* support the role of T-cell activation and corticosteroid-sensitive signaling pathways in predicting future histologic rejection. This clinical investigation is the first to suggest a discriminatory ability to identify early molecular signals that will transform into late allograft rejection or continued quiescence.

Three genes comprising the corticosteroid-sensitive gene set (*IL1R2*, *FLT3*, and *ITGAM*) were lower in peripheral blood mononuclear cells from patients who had an episode of grade 3A or greater rejection within 12 weeks. This reduction is significant in the early posttransplant period, because corticosteroids are the principal immunosuppressive agent subject to active dose reduction within the first 6 months after cardiac transplantation.

Decreased relative expression of five additional genes was observed in the rejection case group, each of which is known to be induced by corticosteroids: *IL1R1*, *TSC22D3*, *FKBP5*, *THBS1*, and *CD163*. This finding indicates a role for lower functional responsiveness to corticosteroids in the increased risk for cardiac rejection. PDCD1, a key cell-surface receptor regulating costimulatory pathways in T-cell activation, was expressed at a significantly increased level in association with future rejection. Six additional genes were expressed at a significantly higher level in the rejection case group: *ADA*, *GZMA*, *TRBC1*, *FLT3LG*, *NFKB1*, and *TNF*. These genes share the property of being transcriptionally induced by T-cell activation or T-cell activating cytokines. These data have the potential to inform clinicians about the balance of immunosuppression, with particular reference to corticosteroid dosing (**Fig. 2**).[11]

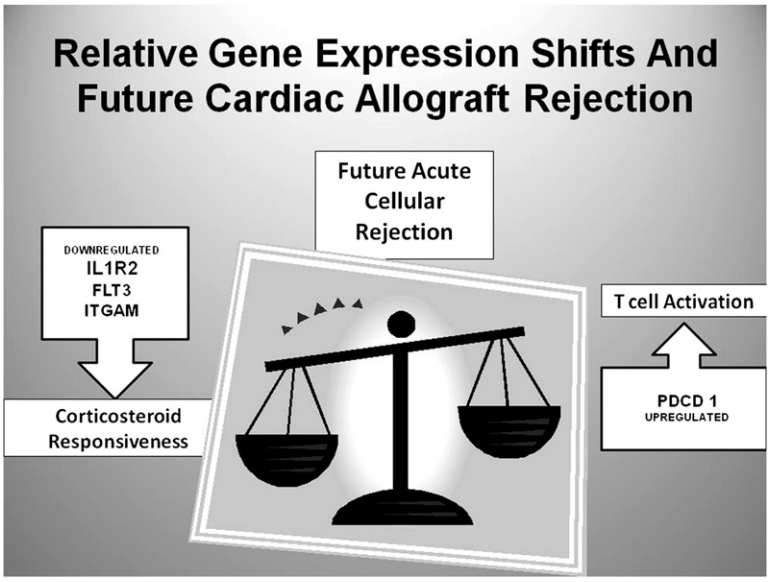

Fig. 2. Predictive molecular gene profiles for risk segregation.

CLINICAL IMPLICATIONS OF TRANSCRIPTIONAL PROFILING

The potential clinical implications of the predictive nature of peripheral blood transcriptional signals prevalent in a cardiac allograft population were shown in a subsequent study by the authors' group. This study showed that 44% of the early posttransplant population (in the first 6 months) will have a gene expression score that provides discriminatory ability to either allocate patients into a low-risk or high-risk group for future rejection.[12] Thus, a gene expression score of 20 or less is associated with very low risk for rejection in the subsequent 12 weeks. In these patients, none (0%) will progress to moderate to severe (grade >3A) rejection, whereas 30% will have follow-up histology that falls within the intermediate group (grade 1B or 2) with unclear implications for treatment. At the other extreme is a gene score of more than 30, which is associated with a 58% incidence of progression to moderate to severe (>3A) rejection.

According to the authors' Bayesian estimates, in aggregate, these two extreme scores ($\leq$20 or >30) are prevalent in 44% of the cardiac transplant population within 1 to 6 months posttransplant. In the low-risk patients, one could develop and validate strategies that include less-frequent endomyocardial biopsies or more-aggressive steroid weaning. Conversely, a higher score points to the need for closer clinical surveillance and caution in aggressive algorithmically ascertained steroid weaning.

LONGITUDINAL CHANGES IN GENE EXPRESSION

Longitudinal gene expression analysis shows that the scores are significantly higher for patients 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 patients in the control group (nonrejectors) show a gradual increase in gene expression scores at the three follow-up time points. This finding is consistent with ongoing corticosteroid weaning, which alters the gene expression score through modulation of the three steroid responsive genes (*IL1R2*, *ITGAM*, and *FLT3*), as noted in the discovery phase of this investigation.

The gene expression has qualitative aspects that show 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. 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 the genes reflecting the erythroid lineage (*WDR40A* and *MIR*), which may denote additional deployment of an erythroid precursor mobilization stress response.[12]

CAVEATS AND PITFALLS OF GENE EXPRESSION PROFILING

Clinicians must develop a working knowledge of transcriptomic profiling, and realize that that these data do not constitute a test with absolute thresholds. First, they must recognize that the multigene panel was developed largely for its negative predictive value. Thus, a threshold of less than 34 (range, 0–40) is associated with absence of ongoing rejection in nearly all patients 1 year after transplantation. However, an elevated score does not mean ongoing rejection; it could mean a heightened risk for future rejection. Second, this panel was not developed in patients exhibiting hemodynamic compromise (a clinically obvious entity) nor has its relevance been established in the unique situation of isolated antibody-mediated or noncellular rejection. Third, the test is invalid immediately after a blood transfusion and in proximity to recently treated rejection.[10]

Thus, clinicians should become accustomed to using cardiac biopsy information as complementary data to those derived from transcriptomic signaling. For instance, a circumstance in which the gene expression test suggests quiescence, but the biopsy seems to represent rejection, should result in a reevaluation of the accuracy of pathologic diagnosis. In these circumstances, the biopsy may have been overread into a higher grade (Quilty phenomenon) or graft inflammation may be nonalloimmune in nature, as with a viral myocarditis, which does not share any common transcripts.

A converse scenario may be a quiescent biopsy with an elevated gene expression score. This scenario could denote a higher risk for future rejection or, in late survivors, indicate cardiac allograft vasculopathy.[10]

COST-EFFECTIVENESS

Cost-efficacy of this approach has been sparsely studied, because nonexperimental use is still in

its infancy. Evans and colleagues[13] calculated that the fully allocated hospital cost for an invasive endomyocardial biopsy, including direct and indirect components but excluding professional fees, is $3297 in the United States. Because a noninvasive monitoring test can reduce biopsy use, it may reduce health care expenditures. Over 5 years, per-patient savings to hospitals, government payers, and private insurers was projected to be $2551, $6550, and $3434, respectively. The overall savings to the health care system can be conservatively estimated at more than $15.7 million annually. These data do not adequately account for the false-positive gene expression score rates that would drive additional unnecessary biopsies, or the scarce but certain occasional cost of handling the consequences of a false-negative gene panel score.

SUMMARY

The advent of gene-based transcriptional signaling in peripheral blood mononuclear cells has ushered in a clinical advance that can not only determine immunologic quiescence but also, more importantly, inform on the future risk for developing allograft rejection requiring therapy. These informative genes represent several biologic pathways, including T-cell activation (*PDCD1*), T-cell migration (*ITGA4*), and mobilization of hematopoietic precursors (*WDR40A* and microRNA gene family cMIR), and steroid-responsive genes such as *IL1R2*, the decoy receptor for IL-2. Using relative levels of gene expression, patients can be segregated into low, intermediate, and high risk for future rejection subsets. This scientific discovery has the potential to allow individualized immunosuppression but requires ongoing independent replication and validation in the real-world context.

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