



GENE EXPRESSION PROFILING FOR THE DIAGNOSIS OF HEART TRANSPLANT REJECTION

A Technology Assessment

INTRODUCTION

The California Technology Assessment Forum has been asked to review the scientific literature on the safety and efficacy of gene expression profiling for the diagnosis of heart transplant rejection.

BACKGROUND

Heart transplantation

Outcomes following heart transplant have improved markedly since the procedure was first performed in 1967. Mortality rates in the year following transplant were as high as 44%.¹ More recently, one year mortality rates in the Registry of the International Society for Heart and Lung Transplantation have decreased from approximately 23% in the 1980's to approximately 15% and continue to steadily improve.² Median survival over the same period has increased from approximately eight years to greater than 11 years.² Acute transplant rejection is a common problem resulting in significant morbidity and mortality. It accounts for approximately 7% of deaths in the first 30 days following transplant, 12% of deaths from day 31 through one year and approximately 10% from years one through three.² Cellular rejection represents the majority of these cases although antibody-mediated or humoral rejection, a different form of rejection that is not easily diagnosed on biopsy, also contributes to morbidity in transplant recipients.³⁻⁵ Much of the improvement in long term mortality is thought to be due to refinements in the immunosuppressive medications that prevent transplant rejection.⁶

After heart transplantation, patients are carefully monitored for signs of rejection. The incidence of rejection peaks at about one month after transplant and then rapidly declines.⁷ Biopsy evidence of rejection usually is present before other signs and symptoms of myocardial compromise, and cardiac rejection is often asymptomatic. Thus, routine surveillance biopsies are the mainstay of early detection and treatment of acute rejection. The usual surveillance course includes endomyocardial biopsy of the right ventricle weekly for the first month, once or twice monthly for six months, and then on an annual basis. Because late

rejection is a rare event, some centers do not perform routine endomyocardial biopsies after one-year post-transplant in clinically stable patients.

In 1990, a consensus conference devised a standard system for evaluating rejection in heart biopsy specimens, the International Society for Heart and Lung Transplantation grading system.⁸ This scale ranges from 0 to 4, with 0 indicating no evidence of rejection and higher scores reflecting greater degrees of lymphocyte infiltration and myocyte necrosis (Table 1). Low grade rejection (ISHLT grade 1A, 1B, or 2) is generally not treated unless there is evidence of a decline in cardiac function. Pulse steroids are usually used to treat higher grades of rejection (ISHLT grade 3A, 3B, or 4) and more aggressive immunosuppressive therapies are reserved for rejection associated with hemodynamic compromise. A revised scale was published in 2005,⁵ but it has not been used in most of the published studies evaluating alternatives to endomyocardial biopsy.

Table 1: ISHLT Grading System for Acute Cellular Rejection

Grade (1990)	Revision (2005)	Mononuclear cell infiltrate	Myocyte injury
0	0	None	Absent
1A	1R	Focal perivascular or interstitial	Absent
1B	1R	Multifocal or diffuse, sparse	Absent
2	1R	Single focus, dense	Present
3A	2R	Multifocal, dense	Present
3B	3R	Diffuse and dense	Present
4	3R	Diffuse and extensive; hemorrhage, edema, and vascular injury may be present	Present

Unfortunately, there is a high degree of inter-observer variability in the grading of the biopsy results⁹⁻¹¹ and transplant rejection can occur in the setting of apparently normal biopsy results.¹² Furthermore, sampling error from random biopsies can miss areas with the most severe/significant rejection. Thus, endomyocardial biopsy is an invasive and imperfect measure of rejection that has risks for significant adverse events. The overall risk of complications is low (<2%), but serious complications, such as cardiac perforation with tamponade, arrhythmias, and death, can occur.¹³⁻¹⁵ Tricuspid regurgitation, occasionally requiring valve replacement, is a long term complication reported in transplant patients who undergo multiple right ventricular biopsies.¹⁶ There is active research attempting to identify less invasive and potentially more accurate methods to identify transplant rejection.

Echocardiographic¹⁷⁻²², electrocardiographic²³⁻²⁵, and MRI²⁶ measures have been studied as early indicators of rejection, but none have proved sensitive or specific enough in validation studies when compared with

endomyocardial biopsy. A number of biomarkers have been investigated in the search for a reliable serologic marker for transplant rejection. These include elevated troponin levels²⁷⁻³⁰, brain natriuretic peptide (BNP)^{31, 32}, c-reactive protein³³⁻³⁶ and soluble interleukin-2 receptor levels³⁷. Recently, gene expression profiling of peripheral blood lymphocytes has generated the most excitement.³⁸⁻⁴⁰

Gene expression profiling

Gene expression profiling refers to a number of different technologies that attempt to quantify the relative levels of messenger RNA (mRNA) for large numbers of genes in specific cells or tissues. The goal is to measure differences in the level of translation (expression) of different genes and utilize patterns of differential gene expression in order to characterize different biological states of the tissue. One potential value of this approach is the identification of genes and gene products associated with a disease process that were not previously known. In cancer biology, the technology has been used to try to differentiate between different subtypes of cancers⁴¹⁻⁴⁷, to identify tumors with good and bad prognoses^{41, 46, 48-54}, and to identify subgroups of tumors with a high likelihood of responding to one therapeutic regimen compared with another.^{55, 56} In transplant medicine, the focus has been on profiling gene expression in circulating white blood cells in order to identify early changes in the immune system that correlate with rejection of the transplanted organ.⁵⁷

The most common approach to gene expression profiling utilizes arrays of DNA sequences bound to a surface like a glass slide. Often, tens of thousands of DNA sequences are organized on an individual microarray in an attempt to profile all of the 20-30,000 genes in the human genome. DNA from a test sample (tumor, white blood cells, normal tissue) is bound to fluorescent dye. Then, it is exposed to the surface of the microarray. Any sample DNA that matches DNA on the microarray (complementary sequences) is bound to the microarray at a specific location. The remaining sample is then washed away. The amount of DNA binding at each site is measured by the intensity of the fluorescent signal. Since the identity of the DNA at each site on the microarray is known, the degree of fluorescence can be correlated with the relative amount of RNA in the original sample.

Another approach to the measurement of gene expression is known as real-time, reverse-transcriptase polymerase chain reaction (RT-PCR). This approach uses the reverse transcriptase enzyme to generate complementary DNA (cDNA) from the mRNA in a sample. The cDNA is then amplified using PCR. This approach is most commonly used to quantify the relative amounts of a smaller set of genes.

Gene expression experiments usually start with microarrays containing many thousands of genes and compare the profiles of tissue with and without certain characteristics in order to identify a smaller subset of

genes that differentiate between the two states (rejection/no rejection; metastases/no metastases). This smaller subset of genes is then validated using new patient samples. Additional candidate genes based on known biological associations may also be included.

These experiments generate tens of thousands of data points, but because of the expense of microarrays and the difficulty obtaining appropriate tissue, the number of patients evaluated is often quite low. Much has been written about the statistical dangers of evaluating thousands of predictor variables in small datasets (multiple hypothesis testing, overfitting).⁵⁸⁻⁶⁰ It is essential that any pattern identified by such experiments be independently validated. Unfortunately, excitement about the results from initial experiments has often overwhelmed statistical caution. One recent paper re-evaluated the data from seven gene expression profiles of cancer prognosis and showed that five of them were likely to predict outcome no better than chance.⁶¹

AlloMap™

AlloMap is the only commercially available gene expression profile currently available for heart transplant patients. The developers of the diagnostic test hypothesized that peripheral blood mononuclear cells may contain information on the host response to the heart transplant and that this could be detected by measuring gene expression levels in these cells. A complex series of experiments (described below) were used to identify eleven genes that distinguish transplant rejection from quiescence. The assay also measures expression levels of an additional nine housekeeping genes that serve as reference standards. Several of the genes are known to be involved in T-cell activation and erythropoiesis, but the function of other genes and their association with transplant rejection is not clear. RT-PCR is used to measure the relative expression of these twenty genes in peripheral blood mononuclear cells. Then, a proprietary algorithm is applied to the results to generate a score ranging from 0 to 40. The value of the score is then used to predict the likelihood of rejection. The exact cut-point for low risk of rejection varies depending on the time since the initial transplant.

Technology Assessment (TA)

TA Criterion 1: The technology must have the appropriate regulatory approval.

Until recently, AlloMap and other Gene Expression Profiles were considered Home Brew tests and exempt from FDA oversight. However, on September 7, 2006, the FDA published draft guidance on planned regulation of In Vitro Diagnostic Multivariate Assays (IVMIA). Complex tests combining data from multiple laboratory tests using a complex algorithm, like those derived from gene expression profiles, will be subject

to FDA review in the future. In particular, those with direct implications for medical therapy will be considered Class III devices and will be subject to the Pre-Market Approval (PMA) process.

The XDX Reference Laboratory (South San Francisco, CA) has received CLIA clearance to conduct the AlloMap test.

TA Criterion 1 is met.

TA Criterion 2: The scientific evidence must permit conclusions concerning the effectiveness of the technology regarding health outcomes. For diagnostic tests, there is evidence that use of the test would result in improved medical management in a way that will benefit the patient.

The Medline database, Cochrane clinical trials database, Cochrane reviews database and the Database of Abstracts of Reviews of Effects (DARE) were searched using the key words 'heart transplantation' and 'gene expression profiling'. These were cross-referenced with the keyword 'human'. The search was performed for the period from 1966 through September 2006 and identified 37 articles. The bibliographies of systematic reviews and key articles were manually searched for additional references. The abstracts of citations were reviewed for relevance, and all potentially relevant articles were reviewed in full. In order to be included in this systematic review, articles had to compare the results of gene expression profiling with the results of endomyocardial biopsies. One evaluated the utility of gene expression profiles using the endomyocardial biopsy tissue itself.⁶² Three publications described gene expression profiles of peripheral blood lymphocytes.^{57, 63, 64} The literature search did not identify any studies that used the gene expression profile to guide patient management. Ideally, randomized clinical trials would compare the clinical outcomes of cardiac transplant patients managed using standard monitoring, including endomyocardial biopsy, to those of patients managed using information from gene expression profiling. However, such studies have not been performed.

Level of evidence: 3

TA Criterion 2 is not met.

TA Criterion 3: The technology must improve the net health outcomes.

The primary outcomes of interest should be overall survival and intermediate measures such as ejection fraction, New York Heart Association functional status, Minnesota Living with Heart Failure quality of life questionnaire, and the 6-minute walking distance. An alternative method to screen for transplant rejection that significantly reduces the need to perform endomyocardial biopsies, while preserving survival and quality of life, would be a great advance. In the absence of such studies evaluating these outcomes, the sensitivity and specificity of gene expression profiling for the detection of rejection using the pathology results of endomyocardial biopsy specimens as the gold standard may be helpful.

Morgun et al⁶² used microarrays to analyze the gene expression profiles of 76 endomyocardial biopsies from 40 patients in Brazil. The biopsies represented 27 cases of rejection or impending rejection, 15 cases of Chagas disease, one patient with leprosy, and 33 quiescent biopsies without Chagas disease. They initially developed a set of 98 genes that discriminated accurately between acute transplant rejection and non-rejection. Then, they repeated the process twice using different subsets of patients to develop two additional predictive models (130 and 188 gene respectively). The 14 genes in common between the three models were then used as the final model. It classified patients with 95% accuracy. They did not perform an independent validation in heart transplant patients, but reported that the same set of genes was able to accurately distinguish rejection from non-rejection in both kidney and lung transplant patients with 94% accuracy. Their work suggested that a common set of genes may be useful to characterize rejection in multiple organs. This approach does not reduce the need for endomyocardial biopsies, but may perform better at the detection of incipient rejection in patient biopsies that do not meet usual criteria for rejection requiring treatment. They note that larger, multicenter studies are needed to refine and validate their diagnostic model.

Investigators in Germany published the first study to attempt to diagnose transplant rejection using peripheral blood mononuclear cells in early 2004.⁶⁴ A total of 58 blood samples from 44 patients were collected on the same day that an endomyocardial biopsy was performed. The samples included 32 diagnosed with < ISHLT grade 2 rejection and 26 with \geq grade 2. The investigators used rt-PCR to amplify the mRNA of 39 genes in peripheral blood that were chosen based on their biological association with processes thought to be associated with transplant rejection. This was not truly an experiment in gene expression profiling, but was an important proof-of-principle step in applying expression profiling to peripheral mononuclear cells. Discriminant analysis applied to the full data set identified five genes useful in differentiating between rejection (ISHLT grade \geq 2) and non-rejection (ISHLT grade < 2): perforin, CD95L, RANTES, COX2, and SEC7/TIC. The authors report that choosing the optimal cut point gave a sensitivity of

82% and a specificity of 84%. They made no attempt to cross-validate or independently validate their results. They note that their study was limited by the small number of patients with Grade 3 rejection and call for studies of larger numbers of genes and patients in order to identify additional parameters to improve discrimination.

Horowitz et al⁶³ reported the first study evaluating gene expression profiling to identify heart transplant rejection. They used an Affymetrix microarray with 22,215 transcripts to compare expression levels in seven patients with ISHLT grade \geq 3A rejection to those in seven patients without significant rejection (ISHLT grade 0 or 1A). The Statistical Analysis of Microarrays (SAM) package was used to select candidate markers for rejection based on a false discovery rate $<$ 10%. Of the 22,215 mRNAs evaluated, 91 met this criterion. The authors further limited the gene set to include only transcripts with at least a 25% difference in expression levels between cases and controls. This left 40 transcripts representing 30 unique gene products. Many important cellular pathways were represented including, transcription, translation, cell cycle regulation, immune response, and apoptosis. The investigators attempted to validate the pattern of 91 genes identified by applying the profile to seven additional samples obtained after treatment of rejection had normalized subsequent biopsy results. The pattern of change observed was consistent with a response to therapy ($p=.0001$).

The Cardiac Allograft Rejection Gene Expression Observational (CARGO) study⁵⁷ was a complex series of studies designed to develop and validate a parsimonious set of mRNA markers that could be measured in peripheral blood mononuclear cells to predict acute heart transplant rejection. The only commercially available test is based on the results of this study. Patients were enrolled prospectively beginning in September 2001. Slides from each patient were sent to a central pathology department for interpretation by a panel of three pathologists blinded to clinical data, though it is not clear if they were blinded to the original interpretation or to the results of the gene expression profiling. Out of 4917 samples from 629 patients, 827 samples from 273 patients were used in the development and validation of the diagnostic test. Patients were required to be at least five years old, at least 21 days post-transplant and post-rejection therapy, and at least 30 days post blood transfusion. The authors included all samples exhibiting rejection (ISHLT grade \geq 3A by at least two of four pathologists) and a “representative” sample of ISHLT Grade 0 samples frequency matched on age, sex, race, use of induction center, use of cyclosporine or FK506, time since transplant, and clinical center. There were three phases to the study. Phase One used a custom microarray to evaluate the relative expression of 7,370 genes in 285 samples from 98 patients. Based on prior knowledge from literature reviews and correlation of gene expression levels with biopsy results, a set of 252 genes was selected for further evaluation. Phase Two used quantitative PCR to evaluate the 252 candidate genes in an additional 145 samples from 107 patients (36 grade \geq 3A rejections and 109 grade 0 quiescent samples).

Phases One and Two were not completely independent. A total of 39 samples from 31 patients were used in both Phase One and Phase Two. Through a complex series of machine learning algorithms, a linear discriminant classifier was developed that utilized 11 genes. The final algorithm gives a score between 0 and 40, with higher scores reflecting a higher likelihood of transplant rejection. Phase Three validated the classifier in two additional sets of samples. The primary validation used 63 samples from 63 patients not included in Phases One or Two of the study. The secondary validation included these 63 samples (31 grade $\geq 3A$; 32 grade 0) and an additional 184 samples, 30 of which were also used in Phase One of the study. The primary objective of the validation study was to test the hypothesis that the diagnostic score distinguishes between quiescence (ISHLT grade 0) and moderate to severe rejection (ISHLT grade $\geq 3A$).

This review will focus on the validation study results. For additional details of the methods used in Phase One and Two, please see the original article⁵⁷ and the supplemental methods that are available online. In the primary validation study, the score from the classifier was significantly higher in samples from patients with at least ISHLT Grade 3A rejection on biopsy compared with samples from patients with Grade 0 rejection (values not reported, $p=0.0018$). The investigators prospectively defined a score ≥ 20 as the threshold for rejection. Using this threshold, the test had a sensitivity of 84% (95% CI 66%-94%) and a specificity of 38% (95% CI 22%-56%). The larger secondary validation set gave similar results (sensitivity 76%, specificity 41%). In a post-hoc analysis, the investigators noted that the scores increased with time post-transplant, usually in association with decreasing the intensity of steroid therapy. The investigators suggest that optimal thresholds should be a score of 28 in the period from six months to one year and a score of 30 for patients more than one year post-transplant.

In an unplanned analysis, the investigators then evaluated the performance of the test in a representative set of 281 samples from 166 patients at least one year post-transplant (prevalent population study). This sample was chosen to avoid spectrum bias: the distribution of ISHLT grade in these biopsies was representative of patients at least one year post-transplant. Only nine of the 281 samples had ISHLT scores $\geq 3A$. Using a threshold of 30, the test had a positive predictive value of 6.8% and a negative predictive value of 99.6%. It is instructive to note that a test that classifies everyone as not having rejection would have a negative predictive value of 96.8% in this test set. Positive and negative predictive values are very sensitive to the prevalence of disease in the population studied. By focusing on a group of patients with a very low probability of rejection, the investigators could be assured of a very high negative predictive value. This says very little about the clinical utility of the test.

The study offers hope that expression profiling of peripheral blood may be useful in some heart transplant patients. However, the results of the primary validation study, using the a priori threshold of 20, were

disappointing. The sensitivity of the test for rejection was 84% (95% CI 66%-94%) and the specificity was only 38% (95% CI 22%-56%). Both estimates had wide confidence intervals reflecting the small sample size in each group. Furthermore, the validation study suffered from significant spectrum bias. Biopsy specimens with ISHLT Grades 1A, 1B, and 2 were excluded from the initial validation study. These grades represent approximately 40% of the specimens used in the prevalent population study described in the paper. Because the AlloMap test measures white blood cell activation, test results are likely to be altered by infection and by the form of immunosuppressive therapy used by the patient. Further studies will need to evaluate the test characteristics of AlloMap in patients with active infection and in patients treated with different immunosuppressive regimens. Finally, in a post-hoc analysis, the investigators realized that a threshold of 20 was too low and that different thresholds were needed for patients at different time points in their post-transplant course. Additional validation studies are needed using thresholds that are defined prior to the start of the study before we can have confidence in the clinical utility of the test.

Finally, it is somewhat disconcerting that none of the 11 key genes in the final CARGO study list match any of the 30 genes identified by Horowitz et al⁶³ or the eight genes identified by Schoels et al.⁶⁴ In a letter to the editor⁶⁵, the CARGO study investigators note that there is some level of concordance with their larger list of discriminatory genes, and that some of the genes in their final set may be the most representative genes in a pathway identified by genes picked out in prior studies. This concern that the statistical methods may simply be very effective modeling of noise in a data set with an enormous number of predictor variables and very few outcomes, underscores the requirement for independent confirmation in a second study.

TA Criterion 3 is not met.

TA Criterion 4: The technology must be as beneficial as any established alternatives.

The established alternative to gene expression profiling is endomyocardial biopsy. Both are used in the context of the patient's clinical history, physical exam findings, and regular echocardiographic evaluation. The right heart catheterization performed as part of the biopsy procedure provides important hemodynamic information, such as cardiac filling pressures and cardiac output, which are also used to guide patient management. These data would not be available to clinicians making therapeutic decisions based on the AlloMap score. No study has been published directly comparing management of patients based primarily on gene expression profiling results to management based on endomyocardial biopsy alone. Ideally, such a study would demonstrate equivalent clinical outcomes with a decreased need for invasive and potentially morbid biopsies. The high negative predictive value of the CARGO prevalent population sub-study suggested that patients at least a year post-transplant may be adequately assessed by gene expression

profiling, but the majority of biopsies have been performed in transplant patients by one year. Some authors have questioned the need for routine surveillance biopsies in this population and some sites do not routinely perform biopsies in patients who are clinically stable. Given the modest positive predictive value of the AlloMap gene expression profile (6.8%), even among patients at least one year post-transplant (93/100 patients with a positive test would not have significant rejection on biopsy), routine gene expression profiling has the potential to increase the number of biopsies performed. This is a significant potential harm that deserves careful attention in comparative trials. Further validation studies need to be performed before gene expression profiling can be recommended in this population.

TA Criterion 4 is not met.

TA Criterion 5: The improvement must be attainable outside the investigational setting.

The large CARGO trial reported data from eight transplant centers suggesting that the sample collection process can be handled reliably at centers with the expertise to perform heart transplants. However, all of the tests were performed at a central laboratory. No data was presented on the reliability of the final test itself. Measures of the consistency of the test results when performed on the same sample would be useful. Given that no improvements in patient outcomes were demonstrated in the studies, TA criterion 5 is not met.

TA Criterion 5 is not met.

CONCLUSION

Heart transplant patients face significant risks for life-threatening rejection, particularly during the first year after transplant. Endomyocardial biopsies are performed according to a strict schedule in order to diagnose significant rejection as early as possible. The search for a less invasive marker of rejection has been a research priority for decades. Gene expression profiling offers the potential for a non-invasive test that may replace endomyocardial biopsy as the gold standard for transplant rejection. However, given the history of poor reproducibility of other gene expression profiles in the recent past, it is prudent to require independent confirmation of the CARGO study results before widespread adoption of the AlloMap gene expression profile to monitor heart transplant patients for early detection of rejection occurs. This is particularly true given the post-hoc change in the threshold used to define a positive test result in the study and the small size of the primary validation study. Additionally, there are no studies published to date comparing the

clinical outcomes of patients monitored with gene expression profiling to those of patients monitored with endomyocardial biopsies.

RECOMMENDATION

It is recommended that the use of gene expression profiling does not meet Technology Assessment Criterion 2 through 5 for safety, effectiveness and improvement in health outcomes when used to manage heart transplant patients.

The California Technology Assessment Forum panel voted unanimously to accept the recommendation as written.

October 18, 2006

RECOMMENDATIONS OF OTHERS

Blue Cross Blue Shield Association (BCBSA)

The BCBSA Technology Evaluation Center (TEC) has not conducted a review of this technology.

Centers for Medicare and Medicaid Services (CMS)

At this time there is not a published policy regarding the use of this technology. However, the lab providing the services does have a provider number.

American College of Cardiology California Chapter (CA ACC)

The CA ACC has been asked to provide an opinion regarding this technology and a representative has been invited to attend the meeting.

ABBREVIATIONS USED IN THIS ASSESSMENT:

mRNA: Messenger RNA

RT-PCR: Reverse-transcriptase polymerase chain reaction

cDNA: Complementary DNA

IVMIA: In-Vitro Diagnostic Multivariate Assays

PMA: Pre-Market Approval

DARE: Database of Abstracts of Reviews of Effects

SAM: Statistical Analysis of Microarrays

CARGO: Cardiac Allograft Rejection Gene Expression Observational

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