Biomarkers as Diagnostic and Predictive Tools in Transplantation: Discoveries at the Bench and Challenges to Bedside Integration

Joyce Kang
Stanford University

Abstract
Solid organ transplantation is a lifesaving procedure performed to treat a multitude of health conditions. Unfortunately, transplant rejection—an inflammatory response mediated by the recipient’s immune system—remains a major and devastating challenge in the field. Because the individual transplant recipient possesses a very unique immunological landscape, much work has gone into making post-transplant care more personalized through the use of biomarkers that can herald rejection before it occurs or diagnose rejection more accurately than standard biopsy techniques. This review paper explores the intersection of science, health, and society with regard to the development of noninvasive and reliable biomarkers in transplantation. The paper examines 1) the motivation and need for biomarkers in transplantation, 2) advancements in high-throughput “omics” technology that have catalyzed recent biomarker discovery, and 3) the complex regulatory challenges in translating scientific discovery at the bench to patient care at the bedside. Through an analysis of the most recently published literature in the field, it becomes evident that the integration of biomarker technology into routine clinical care in transplantation will depend on the concerted efforts of many societal branches, including basic science researchers, technologists, clinicians, industry leaders, and regulatory agencies worldwide.
Introduction
In the field of organ transplantation, striking the appropriate balance when it comes to a patient’s level of immunosuppression is a tricky affair: inadequate immunosuppression may lead to graft rejection, whereas excess immunosuppression increases the risk of infections and cancer. Because the individual transplant recipient possesses a very “unique and dynamic immunological repertoire,” it would be ideal if post-transplant care could be specifically tailored to an individual’s needs (Lo, Kaplan, & Kirk, 2014). This type of personalized care requires the development of noninvasive and reliable biomarkers that could help clinicians accurately gauge the risk of transplant rejection and predict outcomes for individual patients (Lo et al., 2014).

Recent technological advancements in molecular biology and genomics are paving the way for the use of biomarkers as diagnostic and predictive tools for personalized transplantation medicine. The NIH Biomarker Definition Working Group defines a biomarker as any “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson Jr. et al., 2001). Modern medicine currently employs a wide range of biomarkers. For example, cardiomyocyte-specific proteins, such as troponins I and T, are successful as indicators of acute cardiac injury, and plasma glucose levels and hemoglobin A1c levels guide diabetes management (Frangogiannis, 2012).

However, there are currently few suitable biomarkers widely accepted for use in organ transplantation. In the context of transplantation, robust biomarkers would be able to actively monitor immunosuppression and noninvasively detect rejection, allowing for early intervention and enhancing long-term allograft function and life expectancy (Roedder, Vitalone, Khatri, & Sarwal, 2011). This paper reviews some recent scientific developments and controversies surrounding noninvasive biomarkers in solid organ transplantation and discusses some evident challenges in translating discoveries at the bench to routine clinical care.

The Critical Need for Transplant Biomarkers
Solid organ transplantation is a medical procedure used to treat a multitude of diseases. The procedure involves removal of the patient’s damaged or diseased organ—commonly the kidneys, liver, heart, or lung—and surgical engraftment of a replacement organ from a donor. One of the key challenges in the field is transplant rejection, an inflammatory response instigated by the recipient’s immune system. During rejection, immune cells (killer T-cells and activated B-cells secreting antibodies) recognize the transplanted tissue as foreign and mount an attack, damaging the tissue and potentially leading to transplant failure. Strategies to reduce rejection include matching donor-recipient pairs by HLA type (a marker that the body uses to distinguish between self and foreign cells) and using immunosuppressant drugs.
Current diagnostic tools for rejection in solid organ transplantation fall far short of ideal. For example, kidney transplant patients are routinely monitored for changes in serum creatinine levels, which estimates glomerular filtration rate, but since a drift in creatinine level is not specific for rejection (because creatinine production depends on other factors like muscle mass, which can vary with age, gender, and ethnicity), a subsequent invasive tissue biopsy is required for diagnosis of rejection (Kurian et al., 2007; Hernandez-Fuentes & Lechler, 2010). “Histological examination of the allograft biopsy,” in which tissue from the graft is examined under a microscope, is the current gold standard for diagnosing graft function; however, this current approach is suboptimal because it is invasive, is only able to detect rejection at a relatively advanced stage of tissue injury, and completely fails to diagnose subclinical acute rejection, which is rejection in the absence of clinical graft dysfunction (Anglicheau & Suthanthiran, 2008). Furthermore, biopsies are subject to sampling variability and read variability on the part of the pathologist (Roedder et al., 2014). Therefore, there has been a large effort to identify potential ideal biomarkers of rejection that can be obtained non-invasively, such as through the patient’s peripheral blood or urine.

Evaluation of Biomarker Efficacy
What characteristics define an ideal biomarker? In assessing the clinical utility of a candidate biomarker, researchers tend to look at four standard performance characteristics: sensitivity (the proportion of those who have the condition who test positive), specificity (the proportion of those who do not have the condition who test negative), positive predictive value (the proportion of those who test positive who have the condition), and negative predictive value (the proportion of those who test negative who do not have the condition) (Lo et al., 2014). Maximizing these four important characteristics allows for the greatest clinical utility. Together, high sensitivity, specificity, and positive and negative predictive values minimize the rate of false positives and false negatives, thereby increasing confidence that the biomarker can accurately distinguish between groups of patients who either have the condition or not. Figure 1 shows the relationship between the four aforementioned characteristics in the context of a biomarker for rejection.
Technological Advancements and the “-omics” Revolution

Recent technological advancements in high-throughput “omics” techniques, such as genomics, proteomics, and metabolomics, have catalyzed discoveries of novel biomarkers. Genomics focuses on the sequencing and analysis of genes and mRNA transcripts using gene expression profiling (e.g. microarray technology), whereas proteomic analysis characterizes the proteins encoded by the genome using tools like mass spectrometry and protein arrays (Sarwal, 2009). These new approaches demonstrate a fundamental “paradigm shift from hypothesis-driven experiments towards large-scale hypothesis-generating data collection” (Sarwal, 2009).

In other words, instead of having to test individual molecules one by one for their biomarker potential, researchers can now efficiently screen a multitude of possible biomarkers at once, (including genes, RNA transcripts, cellular products, soluble cytokines, and proteins), to identify promising candidates (Kurian et al., 2007). Through modern bioanalytical technologies, researchers can also look at the predictive power of sets of multiple factors analyzed together, such as a suite of multiple genes. “Just as a bar code contains more information than a single number,” the identification of these “molecular signatures” is able to confer much more information than the measurement of a single parameter (Christians, Klawitter, & Klawitter, 2015). Catalyzed by these advancements in technology, many discoveries of biomarkers for solid organ transplantation have been published in recent years.

**Figure 1:** The relationships between each of the four performance characteristics of a biomarker (sensitivity, specificity, positive predictive value, and negative predictive value) in relation to test outcome and actual presence or absence of rejection. Adapted from Lo, Kaplan, & Kirk (2014).
Out of Many, Two FDA-approved Transplant Biomarkers

The application of “-omics” technology to biological samples has generated many potential biomarker candidates; however, “a discouragingly small number make it through the pipeline to clinical use” (Paulovich, Whiteaker, Hoofnagle, & Wang, 2008). In fact, to date, the Federal Drug Administration (FDA) has approved only two biomarkers for use in transplantation: ImmuKnow® (produced by Cyclex) and AlloMap® (produced by the CareDx). The ImmuKnow assay is not specific to one type of transplant—it is used in heart, liver, lung, and kidney transplantation—but instead measures general immune status, either over- or under-activation of the immune system (Roedder et al., 2011). The therapeutic response assay works by measuring intracellular ATP (iATP) levels in CD4+ T-cells (a type of white blood cell that is involved in the immune response), with high iATP levels indicating high immune activation (increased risk of rejection) and low levels indicating low immune activation (increased risk of infections and malignancies) (Roedder et al., 2011).

In contrast to ImmuKnow, AlloMap is a biomarker for rejection specifically in cardiac transplantation. It employs a panel of 20 gene assays, 11 of which are informative and 9 of which are used for normalization and quality control purposes (CareDx, 2016). Using a proprietary algorithm, the gene expression test translates gene expression patterns of mononuclear blood cells from peripheral-blood into a single score ranging from 0–40, representing risk for acute rejection (where the lower the score, the lower the probability of acute cellular rejection at the time of testing compared with patients in the same post-transplant period) (Crespo-Leiro et al., 2015). Prior to the widespread use of AlloMap, cardiac rejection was monitored through an invasive endomyocardial biopsy procedure. Figure 2 shows the timeline of key studies, publications, and milestones that brought AlloMap from discovery in the lab to clinical practice. The initial discovery and validation phase took five years (2001–2005), followed by another clinical trial (CARGO II), leading to FDA clearance in 2008, and continuing studies of AlloMap’s performance are still ongoing (CareDx, 2016).
Although both ImmuKnow and AlloMap help clinicians currently guide post-transplant care, they still have weaknesses as biomarkers. AlloMap possesses a high negative predictive value (one can confidently say that those with low AlloMap scores do not have rejection), but it currently lacks a high positive predictive value (one cannot confidently say that those with high AlloMap scores actually do have rejection), and ImmuKnow has a low sensitivity and specificity (Roedder et al., 2011). Furthermore, a potential drawback of the ImmuKnow assay is that it determines the status of a patient’s general immune function but not necessarily the T-cell reactivity directed toward the graft (Heidt et al., 2011). Therefore, “a new set of biomarkers is desperately needed to replace or complement these tests in order to improve clinical practice with regard to the function of transplanted organs” (Roedder et al., 2011). In particular, since there is currently no widespread biomarker specifically targeted for use in renal transplantation (the most common transplant type), there has been a growing effort to try to identify and develop biomarkers for renal transplantation in particular. The following section highlights some of the recent discoveries in this effort.

Peripheral Blood and Urine Biomarkers for Renal Transplantation

Research into potential biomarkers for renal transplantation has mainly focused on two physical sources of noninvasive biomarkers: peripheral blood and urine. Peripheral blood biomarkers, which can be obtained by simply puncturing a vein and collecting circulating blood, are of particular interest because they can theoretically be applied regardless of transplant type and are easily accessible (Heidt et al., 2011). Many groups have been
investigating peripheral blood biomarkers. For example, Chen et al. employed a fast and low-cost strategy for discovering new diagnostic serum protein biomarkers using publicly available microarray data, finding three protein biomarkers that were significantly higher in acute rejection for renal transplant patients (Chen et al., 2010). Chen’s group’s best marker, serum PECAM1, identified renal acute rejection with 89% sensitivity and 75% specificity, and its increased expression in acute rejection was also confirmed in hepatic and cardiac transplant biopsies (Chen et al., 2010).

According to a review paper by Heidt et al., several research groups have extensively studied perforin, granzyme B, and FasL (molecules involved in cytotoxicity) have been extensively studied as possible biomarkers (Heidt et al., 2011). Up-regulation of two or more of these genes in peripheral blood correlated with acute rejection in kidney transplant recipients with a minimum specificity of 60% and sensitivity of 100%, and further studies have confirmed these results, reporting varying degrees of specificity and sensitivity (Heidt et al., 2011). Researchers looking into post-transplantation levels of sCD30 (a membrane glycoprotein expressed on lymphocytes) as a possible biomarker have found that levels generally decrease, but remain relatively high or increase in the event of rejection, as shown in one cohort of kidney transplant recipients with a specificity of 100% and sensitivity of 88% (Heidt et al., 2011). Other potential peripheral blood biomarkers of acute rejection include mRNA levels for certain cytokines (IL-4, IL-5, and IL-6), as well as other compounds including IFN-γ, TNF-α, and TIRC7 (Anglicheau & Suthanthiran, 2008).

In addition to biomarkers in the peripheral blood, researchers are also interested in urine-based biomarkers for kidney transplantation. Urine is the least invasive clinical sample to collect, and it is also produced directly by the organ of interest (the kidney) and therefore has the potential to be informative in this specific type of transplantation. Unfortunately, the quantification of urine biomarkers can be sometimes difficult and unreliable since urine is produced with highly variable concentration and volume (Hernandez-Fuentes & Lechler, 2010). Among the many urine biomarkers having been studied in kidney transplant patients, the most advanced candidates are CXCL-9 and CXCL-10, which are signaling proteins secreted by leukocytes (a type of white blood cell) in the kidney graft and are inflammation markers that can herald rejection and subclinical infection with sensitivity and specificity far exceeding those of serum creatinine (Christians et al., 2015). Additionally, Aquino-Dias et al. (2008) report finding that mRNA levels of the FOXP3 gene (a characteristic gene of T-regulatory cells involved in acute rejection) in both peripheral blood leukocytes and urine have ~94–100% sensitivity, specificity, and positive and negative predictive values in diagnosing rejection (Aquino-Dias et al., 2008).
Confounding Factors to Consider in Assessing Biomarker Studies

Although a promising number of new biomarkers are being reported in the literature, there are many confounding factors to consider when critically assessing these studies. One of the most important factors that determine success of “-omics”-based biomarker development and clinical implementation is sample quality and consistency in sample processing. However, “in the majority of the relevant published literature, sample collection, handling, and storage procedures are neither appropriately described nor validated” (Christians et al., 2015).

In addition to the sample, other confounding factors in biomarker studies include inconsistent following of protocols and the use of nonstandard analysis algorithms. These factors are best exemplified by a case study involving the report of a promising new biomarker for acute rejection in renal transplantation and subsequent doubts raised about the study design. In November 2014, Roedder et al. from UCSF published a paper in *PLOS Medicine* on a newly developed 17-gene assay, which they called the Kidney Solid Organ Response Test (kSORT), as a biomarker of kidney transplant rejection (Roedder et al., 2014). The biological basis of these 17 genes centered on regulation of apoptosis, immune phenotype, and cell surface. The researchers conducted a large study involving 558 blood samples from 436 samples across eight centers in the U.S., Spain, and Mexico. Using a training dataset, they trained their model and eventually narrowed in from 43 genes to a molecular signature of 17 genes to make up the assay. They then independently validated their biomarker in another set of samples and conducted a cross-validation. In their validation cohort, the 17-gene assay (kSORT) was able to predict 39 of 47 acute rejection (AR) samples correctly as AR, and 87 of 96 No-AR samples correctly as No-AR, resulting in a sensitivity of 82.98% and specificity of 90.63% (Roedder et al., 2014). The authors concluded their paper with great optimism: “the kSORT assay has the potential to become a simple, robust, and clinically applicable blood test” (Roedder et al., 2014).

Soon after the aforementioned study was published, two other prominent researchers—Michael Abecassis, MD, MBA from Northwestern and Bruce Kaplan, MD from the University of Kansas Medical Center—raised several criticisms of the study in a co-authored rebuttal piece published in *Nature Reviews Nephrology*. Abecassis and Kaplan pointed out several weaknesses, mainly the heterogeneous sample (differences in sample handling at different centers), lack of serially collected samples paired with protocol biopsies for confirmation, and insufficient explanation of an analysis algorithm called kSAS that was supposedly used to normalize the data. They even raised a “potential concern that the model will be self-fulfilling” (Abecassis & Kaplan, 2015). Abecassis and Kaplan’s comments remind readers that while many studies on transplant biomarkers are being published, these studies should be evaluated with a healthy dose of skepticism. Clearly, biomarker research presents many
challenges, especially ensuring sample homogeneity across multiple centers and ensuring robust statistical and computational techniques.

Lost in Translation: Challenges in Going from Bench to Bedside

As discussed earlier, the FDA has only cleared two transplant biomarkers, ImmunoKnow and AlloMap, highlighting how difficult it is to translate a biomarker from bench to bedside. The translation process involves a concerted effort on the part of many institutions, with the vast majority of candidate biomarkers eventually being abandoned for one reason or another. This section covers the key steps of the biomarker development pipeline and explores some of the challenges in bringing a biomarker from the laboratory to clinical care.

The first step in the pipeline is the discovery of candidate biomarkers. This is most commonly done in the setting of a single research laboratory or among a few labs in collaboration. Once a potential biomarker has been discovered, the second step involves verification of the biomarker. “The goal of biomarker verification is to determine whether the candidate has sufficient potential for success to warrant investment in time-consuming (and expensive) clinical validation studies” (Frangogiannis, 2012). Verification usually involves a large number of samples to include a wide range of patients and controls, which tends to see a shift in focus from confirming high sensitivity to assessing the biomarker’s specificity (Frangogiannis, 2012). Most candidate biomarkers are eliminated from further consideration after pilot studies performed during the verification phase and careful prioritization (Frangogiannis, 2012). The few surviving ones move on to the third step: the clinical validation stage, which is conducted in a realistic clinical practice environment (Frangogiannis, 2012). This stage is a major bottleneck due to the scope of these large-scale efforts (requires high enrollment of patients at multiple centers) and high cost needed to conduct a comprehensive study. In most cases, the efforts required for clinical validation fall far beyond the resources of a single research group (Christians et al., 2015).

Conclusions and Future Possibilities

In conclusion, diagnostic and predictive biomarkers have the potential to considerably improve the field of solid organ transplantation. Reliable, noninvasive biomarkers could let clinicians tailor immunosuppression in relation to the unique genetic makeup of a patient and their particular biological responses. The “-omics” revolution of recent decades has led to the discovery of many potential biomarkers; however, barriers such as confounding factors in reported studies and the high resource cost of clinical validation preclude the translation of the great majority of these scientific discoveries into routine clinical practice. Despite these challenges, the future for biomarkers in solid organ transplantation looks bright. In 2010, the First International Conference on Transplantomics and Biomarkers in Organ Transplantation was held in San Francisco, bringing
“researchers, technologists, bioinformaticians, clinicians, industry, and regulators from around the world” together with the ultimate goal of advancing the field of transplantation (Sarwal et al., 2011). One key theme from the conference was that in order to bridge the gap between basic research and medical science in transplant biomarkers research, we need a more collaborative and progressive environment among research groups, centers, and stakeholders. Increased collaboration could come in many forms, such as data sharing between research groups, publishing of code used for data analysis, more open discussions at conferences and within publications, and greater cross-talk among centers. The concerted efforts of all key players helps move the biomarker discovery and adoption process one step closer to making personalized care for transplant patients a reality.
References


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