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Biomarkers in Solid Organ Transplantation

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INTRODUCTION

Since the first human kidney transplant in 1954,[1] transplantation has expanded to various organs including heart, lung, liver, pancreas, and even vascularized composites (limbs and face). The outcome for each organ clearly supports the transplantation to be the best management option. Transplantation improves quality of life and is cost effective when compared with other supportive options in end organ diseases[2–12]; more important, it is a life-saving event.

A number of challenges remain, despite the promising data and achievements in organ transplantation. One of the most striking facts is the lack of advancement in long-term graft survival.[13] Although the 1-year survival in kidney transplant recipient has significantly improved between 1989 and 2008, the long-term graft survival did not show much improvement. Although this finding may be attributable to an increased number of higher risk profile donors and recipients in the pool, it underscores the lack of biological and clinical knowledge in long-term graft management. Another concern is the aggravating organ shortage to accommodate increased demand. Based on Organ Procurement and Transplantation Network data as of January 2018, number of patients awaiting organ transplantation has exceeded 110,000 in the United States. Last but not least, the number of therapeutic agents used in transplantation, mainly the immunosuppressive regimen, has been relatively stagnant over past decades.[14] To overcome such challenges, there has been increasing interest in developing novel biomarkers that can guide risk assessment, prognostication, and management.

Recapitulating specific aims in transplantation can help with the systematic categorization of biomarkers. Some of the main goals of transplantation include (1) optimizing allograft and living donor assessment, (2) advancing matching algorithm and immunologic risks evaluation, (3) improving allograft survival, and (4) minimizing unintended side effects from the immunosuppressive regimen. Although certain biomarkers may reveal useful information in multiple domains, a judicious combination of tests is crucial for successful

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outcomes. In this review, we explore the laboratory process and clinical application of selective biomarkers. Finally, we introduce novel biomarkers that were recently discovered and are undergoing validation.

INTRODUCTION OF BIOMARKERS IN SOLID ORGAN TRANSPLANTATION

A biomarker is defined as "a characteristic that is, objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."[15] An ideal biomarker should provide an accurate assessment of the disease status and provide predictive and prognostic value. It should be easy to collect, simple to run the assay, and provide results efficiently and cost effectively.

There is an interesting story behind the first biomarker tested in solid organ transplantation. Before laboratory tests were available to measure alloimmunity in preparation for kidney transplants, full-thickness skin grafts were exchanged between the donor and the recipient to test tissue compatibility.[1] After confirming that there was no evidence of skin graft rejection, Murray and colleagues[16] proceeded with the transplant, and validated the successful graft acceptance. A few years after the first kidney transplant, Patel and colleagues[17] discovered the risk of allograft hyperacute rejection associated with the cytotoxicity of recipient serum (containing antidonor antibody) on donor cells. This revolutionary method is now called a microcytotoxicity test and is the basis for different tests performed in the laboratory. Since then, a number of powerful assays have been introduced for donor–recipient matching and posttransplant management.

TISSUE TYPING AND CROSS-MATCH

During the pretransplantation evaluation, a series of critical tests are performed at the tissue typing laboratory. The assessment begins with the HLA typing of both the donor and the recipient. Luminex reverse polymerase chain reaction sequence-specific oligonucleotide has been a popular method for HLA typing. The Luminex system is based on internally color-coded beads that are in turn coated with various sequence-specific oligonucleotide probes that bind target HLA alleles. When DNA binds, it is subsequently labeled with streptavidin conjugated with R-phycoerythin. Flow cytometry can identify the bead and the presence of amplicon of specific allele as a final read out based on the intensity and characteristics of the signal. Although the resolution for typing is generally lower than that with other techniques such as the sequence-specific primers method or sequence-based typing, polymerase chain reaction sequence specific oligonucleotide is widely accepted as standard practice owing to its simplicity and reproducibility. HLA typing by real-time polymerase chain reaction is more recently becoming a method of choice in several tissue typing laboratories as well.

The more powerful application of the Luminex bead platform is the detection of antibodies circulating in the recipient. The presence of donor-specific antibodies (DSA) correlates with allograft rejection and failure.[18–20] In this US Food and Drug Administration–approved test, the color-coded bead is coated with HLA molecules and incubated with the subject's serum. Anti-IgG antibody conjugated with phycoerythin is then used as a secondary antibody for read out. The Luminex-based solid phase antibody screen has revolutionized the field,

because the information can be registered in United Nation of Organ Sharing's online database system, UNet, for virtual cross-matching. Virtual cross-matching, compared with manual wet cross-matching, drastically decreases the time taken and increases the chance of identifying the best match of candidates with available donors. Virtual matching is essentially an assessment of compatibility of donor and recipient based on a paper report on antigens of the donor and antibodies of the potential recipient. This approach is useful in case of import donors, when the time to perform a wet cross-match is usually not there. Solid phase antibody assay is also performed posttransplantation for de novo DSA surveillance. There are caveats to this assay, because not all antibodies exercise cytotoxicity.

Microcytotoxicity, as discussed elsewhere in this article, is an example of a screening tool for clinically relevant DSA. This discovery has been used for many decades in a format of complement-dependent cytotoxicity, which uses rabbit complement to target donor cell-bound antibodies. However, owing to its technical complexity as well as its suboptimal sensitivity and specificity, series of modifications were made on the Luminex-based solid phase assays. Currently, the C1q assay is a popular assay of choice for detecting complement binding anti-HLA antibodies in patient serum.[21] This flow cytometry-based assay quantifies the recipient's C1q bound to antibodies linked to the donor cell to infer the ability of initiating the classical pathway. Complement-binding antibodies as detected by C1q assay or by other means show higher correlation for acute rejection, antibody-mediated rejection, transplant glomerulopathy, and graft failure when compared with C1q nonbinding DSA.[22,23] In general, it has been shown that high titered antibodies are usually complement binding; this is due to the fact that a high molar amount of antibody is required to recruit complement to mediate cytotoxicity. Not only donor-specific-IgG antibodies, but also donor cell-binding IgM antibodies can be detected with this assay. Although this may be viewed as suboptimal specificity, DSA IgG-negative/IgM-positive patients were found to have antibody-mediated rejection with future development of DSA IgG, suggesting the clinical significance of IgM binding.[24]

ADDITIONAL RISK ASSESSMENT TOOLS

Advances in technology and accumulating data enabled the matching of donors and recipients at the level of epitopes for each of the HLA molecules. Using 3-dimensional modeling, a computer program from the database (HLAMatchmaker) can identify alloantigenic eplets when the high-resolution (4-digit) HLA genotype is provided. Studies have shown an association between degree of epitope mismatch to corresponding risk of antibody-mediated rejection.[25–28] With a dramatic decrease in sequencing cost, epitope matching may become a standard practice in the near future.

Increasing evidence is reported on critical role of non-HLA antibodies in transplantation.[29,30] A number of non-HLA molecules expressed on the allograft display polymorphisms thereby priming the recipient's B cells for alloantibody production in a stressed environment.[30,31] In addition to these non-HLA molecules from the allograft, previously unexposed self-antigens may be uncovered in the setting of inflammation and increase the probability of development of autoantibodies. These antibodies are clinically associated with a higher risk for hyperacute rejection, short-term graft survival, and antibody-mediated

rejection.[32–38] Since the report of antiendothelial cell antibodies,[32] multiple important non-HLA antibodies have been identified. Major histocompatibility class 1–related chain A antigens that share the major histocompatibility locus have been well-described as being related to an increased risk for kidney allograft failure.[39] The presence of anti-major histocompatibility class 1–related chain A antigen antibodies can be tested on a Luminex-based platform. The antiangiotensin type II receptor, which is also associated with an increased risk of recurrent focal segmental glomerulosclerosis,[40–42] can be tested with an enzyme-linked immunosorbent assay. Although these panels are not routinely tested at this time, assays are available at selective transplant centers.

BIOMARKERS FOR ALLOGRAFT MONITORING

Currently available noninvasive tests are generally not able to detect early allograft dysfunction and discriminate rejection from other types of allograft injury. For example, a change in the serum creatinine level is the prototypical alarm for kidney allograft injury to prompt further investigation. Unfortunately, creatinine remains relatively stable until significant damage occurs (not sensitive), and can be increased owing to multiple possible etiologies (not specific).[43,44] In most cases, when clinicians cannot rule out rejection, only a few options are available: an antibody panel to check DSA and a biopsy for histopathology.[45,46] Biopsies have been the gold standard for diagnosing rejection, although the paradigm has been focused on developing accurate noninvasive biomarkers. The approach for identifying a noninvasive biomarker is appealing, because it will minimize the risk and resources associated with biopsying an organ in case of a suspected rejection.[47–51] In addition, novel biomarkers may help to classify different forms of rejection on a molecular basis that will assist in formulating the most effective treatment.[52] In the following paragraphs, we introduce some of the biomarkers that have passed various stages of validation phases. In addition, we briefly discuss potential preclinical studies that may have implications for future biomarker development.

Urine is an attractive source for biomarker mining owing to its ability to be collected conveniently. Urinary biomarkers have high potentials for translation through a longitudinal monitoring method. In addition, the composition is directly affected by graft function in kidney transplantation. One of the most well-validated, noninvasive rejection markers is the urine messenger RNA study by Suthanthiran and Muthukumar.[53] The group isolated RNA from urine cell pellets and tested messenger RNA level of CD3ε and IP-10 with 18S rRNA, markers that distinguished acute cellular rejection from antibody-mediated rejection and borderline rejection. In addition, the signature urinary messenger RNA were elevated before the detection of biopsy-proven rejection, which showed that these markers were predictive. This was a multicenter study through clinical trials in organ transplantation (CTOT-04) consortium, which strengthens the reproducibility.

Urinary proteins were also tested as potential biomarkers. Because chemokines are essential in recruiting inflammatory cells,[54] CXCR3-binding protein CXCL9, and CXCL10 were identified as correlated with allograft rejection.[55,56] Bead-based techniques can be applied again for urinary protein detection, by coating them with antibodies for proteins of interest. Follow-up studies further confirmed the role of CXCL9 in discriminating acute T-cell–

mediated rejection and CXCL10 in antibody-mediated rejection.[57–60] The limitation with urinary chemokines was their inability to distinguish between allograft rejection and BK nephropathy.[57]

The microarray is another attractive platform for testing multiple signature transcriptions and has been mainly used with biopsy samples. A few years ago the molecular diagnostic system was introduced to evaluate the allograft biopsy samples at a gene expression level.[61] This assay interrogated the current limitation with morphology-based diagnosis of acute cellular rejection and antibody-mediated rejection. The group further tested microarray system in a multicenter, large cohort and showed correlations between the selected pathogenesis-based transcript sets and their associated diagnosis showing its superior diagnostic resolution and predictive power compared with the current histopathologic diagnosis.[62,63] The Genomics of Chronic Allograft Rejection (GoCAR) study[64] is another example of a microarray based biomarker discovery, where authors from multiple centers extracted RNA from frozen biopsy samples and tested the differential expression of 13 different genes, and showed a correlation with kidney fibrosis development. The main goals of microarray-based systems are to identify subclinical, at-risk groups and prevent the progression of allograft failure and fibrosis.

A donor-derived cell-free DNA kit (CareDx, Inc., Brisbane, CA) is another ground-breaking technique that has been introduced to the biomedical field. Previously, cell-free DNA has been well-described in fetomaternal genetics and lately in oncology as a novel tool for monitoring circulating tumor signal. The introduction of next-generation sequencing was key to the momentum of this platform,[65] because the cost and time necessary for the assay has now become clinically relevant. In this assay, the donor-derived cell-free DNA detects the frequency of donor single nucleotide polymorphisms and this has been shown to be an effective assay to discriminate rejection in multiple systems. such as the kidney, pancreas, liver, heart, and lung.[66–71] It also showed a predictive value as the level was elevated months before a biopsy-proven rejection event.[71]

Finally, our group developed a platform, an integrated kidney exosomes analysis to rapidly detect kidney allograft rejection with high accuracy using urine sample.[72] Extracellular vesicles facilitate intercellular communication and play a critical role in transplant immunology. As T cells infiltrate kidney tubules during acute cellular rejection, exosomes are shed into tubular lumen with signature membrane protein from parent T cell (CD3 in our study). T-cell extracellular vesicles in urine samples are enriched with magnetic beads coated with anti-CD3 antibody. Captured extracellular vesicles are then labeled with horseradish peroxidase conjugated anti-CD63 antibody, which is a marker used to identify exosomes. For read out, the complex is mixed with a chromogenic electron mediator to generate measurable electronic current. Currently, a multicenter prospective study is being conducted to test the predictive value of this assay in a large cohort of patients.

BIOMARKERS FOR IMMUNE MONITORING

Transplant recipients suffer from infection and malignancy, which stems from the toxicity of long-term immunosuppressive regimens.[73] Clinical trials were conducted to test the safety of

immunosuppression withdrawal in hope of minimizing the burden of potent medications; so far, withdrawal was shown to be associated with increased risk of rejection and allograft failure.[74,75] One's immunosuppression regimen is currently managed according to each center's protocol in an effort to reflect immunologic risks specific to each institution's patient population. In particular, the calcineurin inhibitor, the central component of an immunosuppressive regimen, is titrated based on the serum trough level.[76] However, the target level does not reflect the individual's immune system leading to overimmunosuppression or underimmunosuppression. There are limited methods with which to test immune cell function; therefore, there is an increasing demand for the development of a novel immune monitoring platform.

Interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) is tested to infer the donor memory T-cell activity. The assay quantifies IFN-γ production by mixing isolated recipient memory T cells to donor cells.[77] Elevated IFN-γ production correlates with an increased risk of developing acute cellular rejection and having progressive allograft failure.[78–80] The limitation has been intercenter variation, partly owing to the variation in induction therapy[81] and the technical complexity for standardization.[78] Further optimization with a panel of reactive T cells in place of donor cells, which is often inaccessible after transplantation, and may enhance the usefulness of the ELI-SPOT assay.

When overimmunosuppression is suspected, a US Food and Drug Administration-approved (ViraCor-IBT, Immuknow, Lee's Summit) assay can provide insight on the recipient's immune function.[82] The Immuknow assay exploits the T-cell production of ATP by antigen presentation. $CD4^+$ T cells isolated from the recipient's peripheral blood mononuclear cells are stimulated with mitogen. The induced intracellular ATP level is then measured in a luminometer after adding luciferin/luciferase mixture. A low level of ATP correlates with overimmunosuppression and infections.[83–86] In addition, a single-center, randomized, controlled trial on a liver transplant recipient showed improved allograft survival rate with Immuknow-assisted titration of immunosuppressive regimen.[87] However, different studies failed to prevent rejection based on Immuknow, suggesting technical difficulties with standardizing the test.[84–86,88]

Although current immune monitoring assays stem from T-cell biology, increasing attention has been focused on B-cell function, which is directly linked with acute and chronic antibody-mediated injury.[89] Quantifying DSA generating B-cell function, especially memory B-cell function, may be a sensitive method to predict future antibody production and chronic graft failure.[90] HLA-specific B-cell clones can be detected by HLA tetramer staining, and these B-cell clone frequencies correlated with future DSA detection.[91–93] It is worth mentioning that ELISPOT, which is currently used for memory T-cell function, was initially introduced for B-cell clone detection.[94] Studies have shown the feasibility of detecting DSA-producing B-cell clone with ELI-SPOT.[95,96] Although limitations with clinical translation are expected owing to the rarity and bias in the circulating memory B-cell population,[97–99] functional B-cell monitoring will become an essential biomarker in the near future in conjunction with advancement in B-cell biology.

DONOR CANDIDATE AND ALLOGRAFT QUALITY ASSESSMENT

The accurate assessment of allograft quality during organ procurement and the living donor candidate assessment are essential steps that can affect the expanding donor pool and safety of living donors. At present, assessment is heavily based on crude clinical data, including demographics, medical conditions, cause of death (for deceased donor), candidate allograft function, ischemia time, and postprocurement biopsy on high-risk allografts. However, novel molecular tests are also available to assist risk assessments.

Concern among the transplant community has increased because an increased risk of end-stage renal disease and hypertension was detected in black donors.[100,101] The APOL1 gene variant has been on spotlight as a causal polymorphism for the high frequency of chronic renal failure among African Americans.[102] The presence of 2 high-risk APOL1 alleles has been associated with increased risk of focal segmental glomerulosclerosis and end-stage renal disease.[103,104] Allografts from APOL1 high-risk donors showed a higher frequency of collapsing focal segmental glomerulosclerosis,[105] and young African American donors carrying 2 high-risk APOL1 alleles were identified as the highest risk group for developing chronic kidney disease.[106] Recently, a study was conducted to stratify the donor outcome among black living kidney donors with varying number of high-risk alleles.[107] The study revealed the association of high-risk APOL1 genotype and accelerated estimated glomerular filtration rate loss in donors and is expected to be followed by a large cohort prospective study (APOLLO study). Although there is no current guideline regarding whether or not the APOL1 genotype should be tested routinely, our center counsels on the potential implication of high-risk variation to at-risk group donor candidates while making decision on proceeding with a genetic test. Further study results will guide generating consensus among the transplant society.[108]

SUMMARY

Owing to the complex medical conditions in patients with end-organ disease and the convoluted nature of alloimmunity, biomarkers serve a critical role in transplant medicine. Perhaps we are witnessing the most exciting time in transplant biomarkers—a number of promising biomarkers are being examined at different validation phases and are being introduced in everyday practice, awaiting wide implementation. The discovery of candidate biomarkers has accelerated as a result of advances in science and technology. Examples shared in this review include the donor-derived cell-free DNA test and the APOL1 gene test that would have been impossible without the innovation in sequencing. High-throughput analysis such as single cell analysis[109] and the -omics approach[110] opened a door to discover biomarkers through a hypothesis-generating fashion that complements traditional hypothesis-based experiments. It is imperative for researchers, clinicians, industrial, and administrative bodies to continue to work hand in hand to design an efficient pipeline of biomarkers to address unmet needs for patients.

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KEY POINTS

- Biomarkers in solid organ transplantation are critical tools in assessing immunologic risks and preventing graft rejection.
- A paucity of sensitive and specific biomarkers hinders outcome of both the graft and the recipient.
- Number of novel biomarkers are being introduced; understanding the biological concept and methods can guide effective application of these powerful tools.