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Profiling the Proteome in Renal Transplantation

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Abstract

Improved monitoring of transplanted solid organs is one of the next crucial steps leading to an increase both patient and allograft survival. This can be facilitated through one or a set of surrogate biomarker molecules that accurately and precisely indicate the health status of the transplanted organ. Recent developments in the field of high throughput "omic" methods including genomics and proteomics have facilitated robust and comprehensive analysis of genes and proteins. This development has stimulated efforts in the identification of effective and clinically applicable gene and protein biomarkers in solid organ transplantation, including kidney transplantation. Some achievements have been made through proteomics in terms of profiling proteins and identification of potential biomarkers. However, the road to a successful biomarker discovery and its clinical implementation has proved to be challenging requiring a number of key issues to be addressed. Such issues are; the lack of widely accepted protocols, difficulty in sample processing and transportation and a lack of collaborative efforts to achieve significant sample sizes in clinical studies. In this review using our area of expertise, we describe the current strategies used for proteomic based biomarker discovery in renal transplantation, discuss inherent issues associated with these efforts and propose better strategies for successful biomarker discovery.

Keywords

Urine; Proteomics; Peptidomics; Biomarker; organ transplantation; Acute rejection; Non-invasive biomarkers; Biomarker discovery

Introduction

Need of effective and non-invasive biomarkers for renal transplantation

Biomarker discovery and validation is one important aspect of translational research. Even though recent advancements in immunosuppressive therapy has improved the short-term outcome of transplanted organs, acute rejection (AR), and chronic allograft injury (CAI) still remain significant risk factors for allograft failure [1-2]. The current method of allograft monitoring is sub-optimal where a rise of serum creatinine is nonspecific, occurring only with significant pathologic injury that is driven by a wide range immune and non-immune mechanisms, and conversely, early graft injury can occur in the absence of a significant elevation in the serum creatinine [3]. The alternative monitoring method and current diagnostic gold standard, the renal biopsy, is associated with a number of complications, which include pain, sedation, hematuria, arteriovenous fistulae, graft thrombosis, transfusion risk and potential allograft loss [4]. A relentless effort has been put in the field of biomarker discovery using high throughput gene expression analysis (microarray) and proteomics since the publication of human genome data [5] (Figure 1A). Recent advances and sophistication

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in sample preparation methods, mass spectrometry, bioinformatics, and data analysis tools have prompted an increased number of efforts in identifying potential clinically useful protein biomarkers in kidney transplantation (Table 1). Unique and cutting-edge proteomics technologies integrated to identify candidate protein and peptide biomarkers present in the urine, blood, and kidney biopsy of renal transplant patients with specific etiologies of graft injury helps in screening for specific types of graft injury. These biomarkers are expected to distinguish different graft injury phenotypes indistinguishable by the serum creatinine, and may assist in early directed treatment to mitigate tissue injury progression and fibrosis. Still, there are hurdles and challenges to identify the ultimate biomarker and use them in the clinical setting[6]. Several issues in the field of proteomic biomarkers in kidney transplantation still remain, including sample size, well collected and annotated samples, use of sophisticated instruments, and data analysis methods [7-8]. In this review, we have presented an up-to-date summary of the application of proteomic methods and associated issues in the field of kidney transplantation to identify effective biomarkers.

Biomarkers for Kidney Transplant and Current Proteomic Strategies

Biomarkers in Kidney Transplant

One of the critical research issues in the field of transplantation is the identification of clinically applicable biomarkers that are feasible for broad spectrum clinical screening and diagnosis. These biomarkers would help assess an individual patient's risk for acute and chronic allograft damage, allograft tolerance and monitor immunosuppressive therapy efficacy. The current strategies, such as the serum creatinine and measurement of immunosuppressive drug levels have not been precise and accurate enough to assess the risk of drug toxicity and predict allograft rejection. In replacement of these, several immune monitoring assays have been studied over flast decades, specifically focusing on adaptive T cell activity and innate immunity, such as ELISPOT [9] and ImmuKnow assay [10], have seemed to be promising predictors of immune response and outcome of transplanted graft. However, none of these tools have yet been considered in clinical practice. Prediction and early detection of alloimmune specific and non-specific injury processes prior to the change of clinically observable parameters is of prime importance, thus providing the necessary information and early warning to the physicians before significant injury occurs to the graft, essentially a window of opportunity for clinical intervention. Identifying such parameters (genes, mRNA transcripts, microRNAs, peptides, proteins, and metabolites) has been investigated by monitoring these parameters in allograft biopsy, blood and urine. To date, several of such potential biomarkers have been reported, yet no novel biomarkers have been validated in a large multicentre clinical trial for either clinical practice or drug development. Research performed in recent years has yielded a number of minimally or non-invasive biomarkers in the field of kidney transplantation. One such study using gene expression analysis from Vasconcellos *et al.* [11] reported an increased expression of T cell transcripts such as granzyme B, perforin and FasL at the time of acute rejection in peripheral blood mononuclear cells (PBMC). Increased expression of these genes at the time of acute rejection has been validated, but the increase of expression is found to be associated also in case of CMV infection, UTI and delayed graft function by subsequent studies performed in the blood and urine [3, 12-13]. Renesto *et al.* have identified an increased expression IFN gamma inducible genes (e.g. CXCL9, CXCL10, CXCL11) in urine[14]. Schaub *et al.* have reported an increase of CXCL9 and CXCL10 protein in the urine of patients with acute interstitial inflammation and tubilitis in both subclinical and clinical acute rejection [15], who also identified β2-microglobulin (β2-m) for acute rejection in a separate study [16]. In addition to β2-m, retinol-binding protein (RBP) [17] and α1-microglobulin (α1-m) [18] are elevated in the urine of patients with acute rejection, neutrophil-gelatinase-associated lipocalin (NGAL) has been reported as a marker of graft recovery after kidney transplantation [19]. In a recent study, we have reported an elevation of UMOD, SERPINF1,

and CD44 in the urine of patients with active acute rejection [20]. With a novel integrative strategy that utilized both gene expression and peptidomics data, a 40-peptide panel including UMOD and collagen peptides has been reported to be acute rejection specific in urine of kidney transplant patients [21]. Using a bioinformatics approach across different solid organ transplants (kidney and heart) Chen *et al.* identified a number of proteins as potential biomarkers for acute rejection that were successfully validated by ELISA [22].

Strategies for Biomarker Discovery in Kidney Transplantation

The direct involvement of proteins and peptides in a number of various biological and pathological processes makes it quite relevant to identify and quantitate their profile in relation to injury development and progression. The understanding gleaned from proteomic and peptidomic analysis not only provides a potential surrogate biomarker to early detection of acute rejection and injury progression of the allograft but also monitor drug efficacy in maintaining graft health. Several proteomics approaches have been utilized to profile the entire spectrum of the proteome [23]. The whole-expression proteomics approach to proteomic profile, takes advantage of the high-throughput nature of the current technology platform to characterize the differential protein expression between normal and disease states. Like the microarray based gene expression assays, proteomic research in the field of transplantation has increased in recent years, as reflected by the growing number of publications (Figure 1A). In the field of kidney transplantation gel-free methods such as SELDI-TOF, CE-MS, and LC-MS/MS have remained the most popular method used, especially for urine samples. Peptidomic approach and protein microarrays also seem to be promising tool for the new biomarker discovery, as these offer highly sensitive analytical capabilities with large dynamic range of detection geared towards identification and quantification of proteome changes [24].

To identify proteins for discovery purposes, LC/MS based Shotgun Proteomics has shown the most promise because of its ability to identify more than a thousand proteins from healthy urine [20, 25]. In a recently published report, we applied shotgun proteomics to analyze a set of urine samples, from patients with AR, stable grafts (STA) and controls (HC) that led to an identification of a total of 1446 urinary proteins. Using semi-quantitative protein-level spectral counts, we identified a number of AR specific urinary proteins. This first of its kind study not only identified novel proteins in the urine of renal transplant patients but also helped to elucidate the increased presence of proteins related with MHC antigens, complement cascade and extra-cellular matrix. The main stumbling block with proteomic studies thus far are that they have been based upon a relative small cohort of patients, where a comprehensive and rigorous analysis of the non-diseased or ‘normal’ urinary proteome would establish a threshold for urine protein concentration across the complete human population. In addition, as with other high throughput methods, the platform needs to be carefully controlled for run-to-run variation and variations in performance efficiency of both LC and MS [26]. In a recent report Nagaraj and Mann used LC MS/MS to analyze urine collected from 7 normal human donors for 3 consecutive days and reported that there are about 500 urine proteins that can be referred as common and abundant in human urine[27]. This type of work that establishes a minimum threshold and explores on the variation within individuals, platforms and runs will help in validating and consolidating urine proteomics data towards clinical application.

Peptidomics of endogenous urinary peptides brings another possibility that it may provide a tool to examine thousands of small MW endogenous peptides top-down approach. This approach identifies peptides in the molecular weight range of 900-5000 Da by tandem mass spectrometry (MS/MS) and can yield the identification of thousands of peptides in a single experiment [28-29]. These peptides could be used as clinically useful biomarkers for different renal and systemic diseases as they are product of proteolytic degradation [30]. In a

method developed within our lab we prepared native peptides for analysis by performing the following steps: (i) extraction of peptides, (ii) fractionation of extracted peptides using HPLC, and (iii) MS analysis of fractionated peptides for biomarker discovery purpose. Different strategies could be applied to fractionate the mixture of peptides. First, a pilot study with pooled samples established feasibility of this approach [30], where we were able to demonstrate an AR specific repertoire of endogenous peptides that were differentially present in AR urine. To further investigate on the power of endogenous peptides we expanded the study to 70 unique samples from 50 renal transplant patients and 20 controls. Using LC-MALDI approach we identified a specific panel of 40 peptides for acute rejection (AR) that successfully differentiated AR from other phenotypes, including stable graft function and BKVN [21]. In a separate report, Quintana *et al.* [29] analyzed a total of 71 urine samples collected from 39 patients with chronic allograft injury and 32 control individuals. The study identified several peptides from proteins such as uromodulin as differentially present in the patients with chronic graft injury. Peptides with m/z 638.03, m/z 956.56, and m/z 1003.62 as significantly decreased in chronic allograft injury as compared to control patients. Peptidomic approach has also been performed using CE MS. In a recently published report Metzger *et al.* identified and sequenced 20 peptides specific for acute kidney injury (AKI) [31]. With its fast separation ability and its robustness to analyze thousands of polypeptides within an hour, CE-MS has been utilized in a number of studies[32]. All of the aforementioned peptidomics methods still need to be assessed on their performance across hundreds of samples, which will require rigorous testing in a multicentre trial for any of the identified potential biomarker peptides to be approved for clinical diagnostics [33].

SELDI-TOF has been applied in studying AR specific [16, 34-35] and BKVN specific biomarkers [36]. Several peptides including β2-m peptides were found to be specific to AR. A study by Jahnukainen *et al.* (2006) identified 5 peptides that were specific to BKVN. Despite the benefit of SELDI as a relative low time consuming method for peptidomic analysis, thus facilitating high through, its low resolution “mass fingerprinting” and inability to identify corresponding peptides has resulted in low enthusiasm towards this strategy of biomarker discovery.

Gel-based proteomics allows evaluating intact proteins in an unambiguous manner, which is readily available and inexpensive, resolving proteins in two dimensions based on their isoelectric point (pI) and molecular weight. Proteins of interest can then be excised and proteolytically digested by trypsin, and identified by tandem mass spectrometry [37]. In 2D DIGE, two samples are labeled with two different fluorescent dyes (e.g. Cy3 and Cy5), which are then mixed with an internal standard and applied to a single 2D gel. This approach has been reported to reduce gel-to-gel variability [38-39]. This technique could be useful to optimize urine sample processing for proteomics analysis [8]. This technique was successfully utilized Banon-Maneus *et al.* to identify biomarker urinary proteins associated with chronic allograft injury (CAI). The authors were able to demonstrate the DIGE technology can differentiate patients with different IFTA grades with a possibility of these proteins being candidates for a larger prospective validation study for biomarker validation [40]. However, a gel-based approach suffers from its detection limit, thus only allowing the identification of a limited number of proteins that otherwise would have been identified by LCMS based shotgun proteomics. In addition, the high degree of variability in urine concentration capacity also causes high inter-individual variability (among individuals) and poor intra-individual reproducibility (within the same patients). These limitations have hindered the wide use of gel-based approach in transplantation research (Table 1).

Protein Arrays—Alloantibodies play major roles in acute and chronic rejection of transplanted organs and can provide an important milieu for biomarkers. Not much is known

about their role of these alloantibodies in causing graft injury in different immunosuppressive environments. Binding of alloantibodies to HLA or other nHLA targets in the graft or the graft microvasculature can initiate activation of the immune complement system, recruitment of leukocytes and potentially facilitation of monocyte/macrophage or NK-cell mediated cytotoxicity. Antibody formation may, like heterogeneous T cell immunity, relate to immune exposures that are not primarily donor HLA-specific. The current standard of diagnosing antibody mediated rejection relies on the demonstration of C4d staining within the graft and identification of HLA-Ab in the circulation. Nevertheless, C4d staining is relative insensitive as it is recognized that humoral mechanisms of graft rejection can occur in the absence of both positive C4D staining and the presence of detectable circulating HLA-Ab, which may in fact occur secondary to circulating alloantibodies to nHLA antigens, the repertoire of most of these being currently unidentified. Minor-histocompatibility antigen specificities may be involved in targeting currently unapparent genetically determined differences. Although the precise number is not known, it has been estimated that thousands of coding single nucleotide polymorphisms (SNPs) occur within expressed exons resulting in amino acid polymorphisms in thousands of common cellular proteins. The vast majority of coding SNPs are functionally inconsequential, but represent targets for nHLA-Ab formation. Recently, microarray technology has evolved beyond that supporting nucleic acid hybridization to serve as a platform for detecting protein-specific antibodies [8]. Proteinarray technology, which has recently been used by us and the others, allows for serologic screening for reactivity against thousands of defined human proteins to identify antibodies against nHLA antigens [41-43]. We found that nHLA-Ab can be induced during alloimmune responses and mediate graft damage, and new onset nHLA-Ab can be detected in patients undergoing allograft rejection independent of HLA-specific antibody responses. Evaluating differences in the post-transplant generation of these antibody responses in association with developing acute and chronic graft injury, and determining the specificity of these antibodies responses to kidney and viral antigens can be an interesting avenue in the field of renal transplantation.

Issues in Biomarker Discovery Efforts and the Solutions

There are several barriers to the success of the biomarker discovery and proteomic analysis. The difficulty in applying proteomics in the clinic is largely attributable to the wide dynamic range of protein abundance, complexity of clinical variables and possible variability between samples due to the method of sample handling.

High abundant proteins and Fractionation

The evolution of current MS-based proteomic approaches have contributed substantially to our understanding of the molecular characterization of blood and urine proteins. Different tissue types are relevant for different organ types for biomarker studies. However, one of the main hurdles to biomarker discovery with blood and urine proteomics is discerning proteins present at low levels from highly abundant proteins such as albumin and uromodulin. Proteomic analysis in all clinical samples is hampered by its complexity. In serum, ten high abundant proteins, such as albumin and immunoglobulin, account for more than 95% of the total protein [44]. Therefore, removal of these major abundant proteins is mandatory to allow detection of the remaining lower abundant proteins.

Since, urine reflects local events within the kidney and changes within circulating blood, urine is the most relevant biofluid to be screened in the case of kidney and urological diseases and has been preferably analyzed over blood or kidney biopsy samples[45]. In urine proteomics, major abundant urinary proteins obscure the identification of low abundant proteins as seen in microarray analysis of blood samples. In addition, different origins of urine proteins add to its complexity, and its composition is inconsistent with normal

physiologic state to various disease conditions. The origin of protein in proteinuric patients could be one of the following; first, the filtrate of plasma protein through intact (overflow proteinuria) or damaged filtration barriers (glomerular proteinuria), second, impaired reabsorption of the filtered protein caused by the tubular injury, third, secreted protein from the renal tubule such as THP, fourth, proteins originated from injured kidney tissue (nephrinuria in diabetic nephropathy, tubular enzymuria in acute kidney injury), finally, excretory vesicles (exosomes) or membrane-shed vesicles (microparticle, also referred to as ectosomes) from kidney and uroepithelial cells. Simple elimination step of high abundant proteins would not be a solution to identify the pathogenic specific protein markers among this complicated mixture of proteins of from different origins. Known fragmentation pattern of abundant proteins and proteolitic activity in nephritic syndrome and oxidation property of plasma albumin in FSGS suggest that these abundant proteins also could be informative in biomarker discovery [46-48]. And the elimination step of high abundant proteins could be another confounder in “quantitative” data analysis and can cause the loss of other biologically relevant proteins. The removal of major abundant proteins should be chosen after considering the types of samples and study design and its advantage should be further elucidated.

One of the strategies that can be implemented in analyzing urine for efficient search of biomarkers is to fractionate different compartments of urine sample. Blood samples can be fractionate and analyzed to dig deeper into the blood proteome. Urine contains a mixture of analytes. In order to maximize the discovery power urine can be split into different fractions as elucidated in Figure 2. As urine contains cells, exosomal vesicles, intact proteins, native peptides, and metabolites, a variety of molecules can be analyzed by different high throughput platforms.

Clinical confounders and normalization

In any clinical study, it is essential to define the patient and control populations. The outcome of the study depends on how well the patients’ phenotypes are defined. The spectra of defined diseases are often too broad with samples reflecting different disease etiologies all affecting the outcome of biomarker discovery significantly. Underlying disease before transplantation, co-morbid diseases, used medication and degree of immune suppression can also have significant effect on the biologic specimens and subsequent proteomic data. Control samples from normal healthy individuals are often not suitable to compare the proteomic data from the specific category of transplant patients [49]. To find the disease specific biomarker rather than “non-specific injury marker”, transplant patients with similar clinical, biochemical and metabolic profiles should be added to the disease controls.

Apart from other biologic samples, intra-individual and inter-individual variability’s are still most difficult problems in proteomic data analysis [50]. Because urine protein concentration is affected by the wide range of urinary concentrating and dilution mechanisms of renal tubules and collecting duct, interpretation and quantitative analysis of urinary proteome data require a normalization step. Most of urinary proteomics studies still use the relative amount of specific protein compared to the total protein amount. However, degree of proteinuria in transplant patients is widely variable, and it can originate from the heterogeneous causes as mentioned above, which in turn are affected by non-specific kidney injuries as well as non-kidney diseases. Other normalized methods for urinary proteomics are 24-h excretion, rate of excretion within a certain period of the time, and normalization with urine creatinine abundance [51-52]. However, timed collections of urine are inaccurate and not easy in clinical application, with the use of creatinine inadequate due to the high subject-to-subject variability and impact from renal injury, as described previously, in its rate of excretion [53], leaving the issue of normalization in urine proteomics to be further elucidated.

Sample related confounders

A wide range of sample related variables should be defined to ensure the usefulness and comparability of the results of the proteome analysis. They include the appropriate selection, processing, handling and storage for each biologic sample, all of which may markedly influence the final results obtained, but are often overlooked. As compared with other biologic samples, urine is considered to need more sophisticated approach for sample handling and collection when used in proteome analysis, because urine has a low and highly heterogeneous protein milieu, high levels of salts, a number of interfering compounds, high degree of variation in its concentration all combined with hematuria, bacterial contamination, and the presence of proteases. Many researchers have investigated and recommended a number of different appropriate handling of urine samples, where we have also compared the benefits and shortcomings of different protein recovery methods, to which we recommend the depletional strategy in urine proteome approach [6, 54-55]. In order to create a so called “universal standard” a group of researchers worked on to develop a well-characterized “real life” sample that can be used as reference standard in urine clinical proteomics studies [56]. The authors have made this control urine available and believe that it will provide a standard for the comprehensive characterization of the urinary proteome. Still, several issues, including sample collection, removal method of high abundant protein, use of protease inhibitor for proteinuric urine still remain issues to be addressed.

Robust identification and quantification

Effective proteomics study relies on the ability of protein identification and quantification. One of the challenges noticed earlier in blood and urine proteomics was to overcome the barrier of high abundance proteins, which has been solved with the help of fractionation and depletion strategies [57-58]. As noted in an earlier section of this review, different strategies have been adopted to analyze urinary proteome to date [59-64]. It started off with gel-based techniques that identified relatively fewer proteins than gel-free LC-MS approach. Adachi *et al.* identified 1492 proteins using urine collected from healthy individuals [63]. In a recent report, Gonzales *et al.* have identified 1160 from human urinary exosomes [64]. In one of our previously published studies, we identified a total of 1446 proteins with stringent criteria of a minimum 2 unique, non-redundant peptides per protein with ~0.1% FDR for protein identification. A successful biomarker discovery using proteomics would not only require identification of peptide and proteins but also an ability to quantify the analytes. One challenge posed during this kind of work is that there is approximately as high as 10 orders of magnitude of protein concentration variation in the biofluids such as blood and urine. In our study we were able to identify high abundant proteins such as UMOD with a concentration measured 5 orders of magnitude (~0.07 mg/mL) more than the concentration measured for protein S100 calcium binding A4 protein (~2 ng/mL) in urine [65]. In this study, spectral counts were used as a semi-quantitative means for comparison and a weighted fold-change was used for potential biomarker listing. When we tested 3 proteins whose concentration was differed by 4 orders magnitude there was a nearly perfect correlation to a good correlation of the proteins as quantified by ELISA (r^2 = 0.59-0.99). Our data suggested that label free LC-MS/MS spectral count data provides relatively good quantitation for high abundance to moderate abundance proteins [65]. In cases where the spectral count is very low, this method of quantification produces a poor correlation with the real concentration in the sample and, thus may require more stringent labeling methods such as iTRAQ [66] or $^{18}O/^{16}O$ labeling method [67] to achieve an accurate quantitation.

In order to make a substantial progress towards helping renal transplant patients with a non-invasive clinical tool to monitor renal transplant, it has become necessary for the different transplant centers to come forward and collaborate in sharing the data that have been

collected through different projects. If individual centers contribute their high throughput gene expression, proteomics, and protein array data to a public repository, it might culminate into a Biomarker Database Server that can be made available to all researchers. A schematics of building of such a data base is shown in Figure 3.

Biomarker Validation

The proteomic studies in the field of renal transplant thus far have only focused on preliminary list generation of potential biomarkers [16, 21, 30, 35, 65, 68]. This is in part because these studies were first of their kind and served as proof of principal in demonstrating the utility of these proteomic based methods. Given, that this is a significant step forward, however simple protein/peptide-disease association is not enough and requires the extensive validation of these potential biomarkers identified. Two factors play critical role in validating these potential proteins and peptides to make them candidates for clinically useful biomarkers. (1) *A need of larger sample cohort in validation studies*. Any biomarker molecule that has a potential to go to clinical trial must be verified and validated in a large independent cohort of samples and patients [69-70]. The critical time has arrived where biomarkers discovery efforts must focus on cashing in on the investment that was made in discovery efforts. Given the rate of kidney transplantation that happen within individual hospitals, and with the declining rejection rate, in order to give enough power to the discovery, researchers would need to establish a collaboration to utilize urine samples collected from different centers. (2) *A need of integration of information gleaned from high throughput analyses such as gene expression, proteomics and protein arrays*. With more and more gene expression, proteome, peptidome, and protein array data being collected from samples collected from renal transplant patients it has now become possible to integrate the data and use a novel strategy to get to useful biomarkers and achieve better understanding of transplant dysfunction. In a recently published work by Li *et al*. used publicly available microarray data and used protein array data to identify non-HLA antigen targets after renal transplantation [41]. In a recent report published by Chen *et al*., the authors used novel bioinformatics strategy to integrate microarray data to screen for potential biomarker proteins which could be validated in the serum by ELISA method. The beauty of these novel strategies is that they could even identify markers that are part of the common rejection mechanism across different solid organ transplant fields [22] (Figure 4).

Personalized Medicine in Kidney Transplant

Cellular and humoral immunity are considered as a common pathway of immune response to the transplanted organ [71]. However, the injury is far from simple and is a complicated process that is a result of the interplay between different immune related pathways that is specifically altered in different organ recipients. The multifactorial nature of graft dysfunction has added to the complexity of graft monitoring and treatment for transplanted kidney dysfunction. Tailoring patient care based on individual needs as opposed to current “one size fits all” approach is one of the challenges for scientific community in post-genome era[72-73]. Pharmacogenetics, the incorporation of genetic data into the assessment of drug dosing, response and adverse effect, is an important challenge to provide the patient-oriented individualized immune suppressive regimen in transplantation medication. Recently, the associations of several polymorphisms in CYP3A, ABCB1, TPMT and ABCC2 genes that affect requirement of common immunosuppressive agents in solid organ transplantation have been studied [74-77]. However, some drug related polymorphisms are just a small piece of the whole panel of drug metabolism in transplantation and pathogenesis of graft dysfunction. Integration of transpcriptomics and proteomics into more extensive genetic data is more promising and may be incorporated into the treatment algorithm when it is combined with more complex clinical parameters. High throughput DNA sequencing is a powerful tool to perform genomic, epigenomic and transcriptomic studies. It allows the

determination of large numbers of short sequences (30-40 million reads 36-100 bp in length for an Illumina GAIIx) from one or both ends of DNA fragments in a high throughput manner [78]. Methods such as RNA-Seq enable precise monitoring of distinct splicing isoforms to acquire single-base resolution in a high throughput manner that can be used to screen for individual gene variants in between the donor-recipient pair [79]. Based on the information gleaned from DNA or RNA sequencing, donor specific antigenic targets can be identified which can be utilized to monitor the alloresponse of the recipient by assaying antibodies targeted to donor specific antigens.

Conclusion

Noninvasive reliable and sensitive methods to diagnose rejection and other forms of injury to the transplanted organs, including kidney, are currently unavailable. However, the initial utilization of proteomic approaches to identify potential protein and peptide biomarkers in kidney transplant has been encouraging. This preliminary success, however, needs to move on to validating potential biomarkers by rigorous validation steps utilizing even more sophisticated techniques that have recently become available. Due to the lack of sufficient patients in one lab or center, this can not materialize unless researchers from different states and countries collaborate in this noble cause of validating clinically useful biomarkers that will eventually benefit the patients ailing in the hospital beds. It is therefore a high time for a team of experts from the field of transplantation medicine, genetics, immunology, molecular biology, pharmacogenomics, biostatistics, and bioinformatics to work together for a meaningful improvement in patient care.

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Abbreviations

AR	Acute rejection
STA	Stable graft function
CAI	Chronic allograft Injury

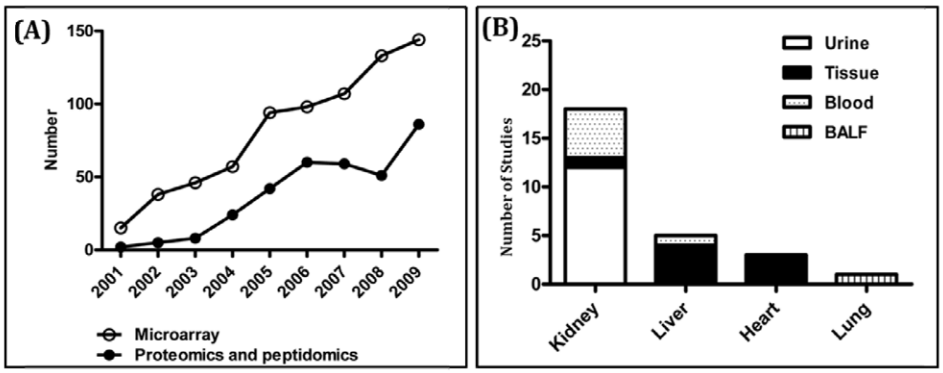

Figure 1.

Trend of application of proteomics in the field of solid organ transplantation. (**A**) Number of studies using in the field of solid organ transplantation using high throughput methods are on the rise. Even though microarray gene expression analysis still surpasses number of works in the proteomics field, there is a trend of increased number of proteomics studies in the field of transplantation. (**B**) Majority of the proteomics studies in renal transplantation are performed in urine followed by blood. Liver and heart proteomics are performed with tissues where as BALF has been used for lung transplantation.

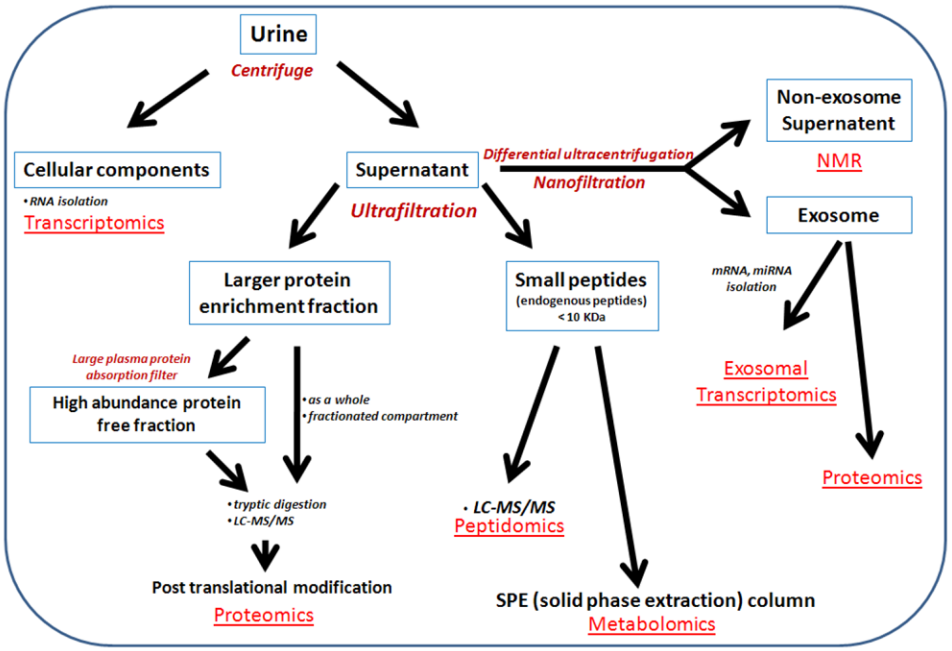

Figure 2.

A strategy of analyzing urine for efficient search of biomarkers. Divide and analyze has always been proved to be effective to maximize the power of discovery by analyzing low abundant molecules. Urine having cells, exosomal vesicles, intact proteins, native peptides and metabolites presents a variety of molecules to be analyzed by different high throughput platforms.

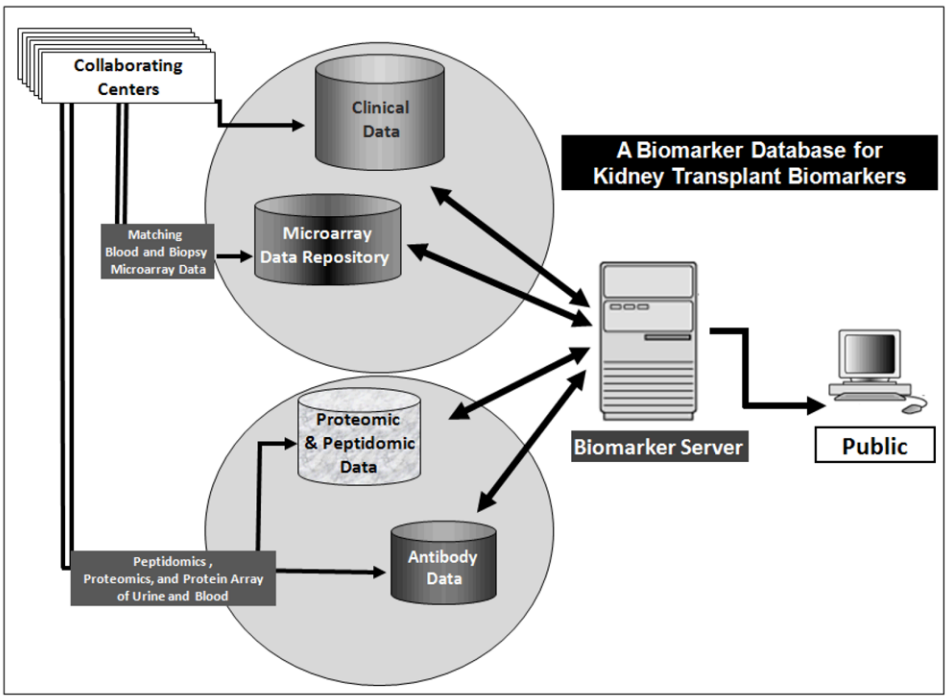

Figure 3.
A schematics of proposed “Biomarker DB” for kidney transplant which could streamline discovery and validation studies and assist in identifying a very specific and non-invasive clinically useful biomarker.

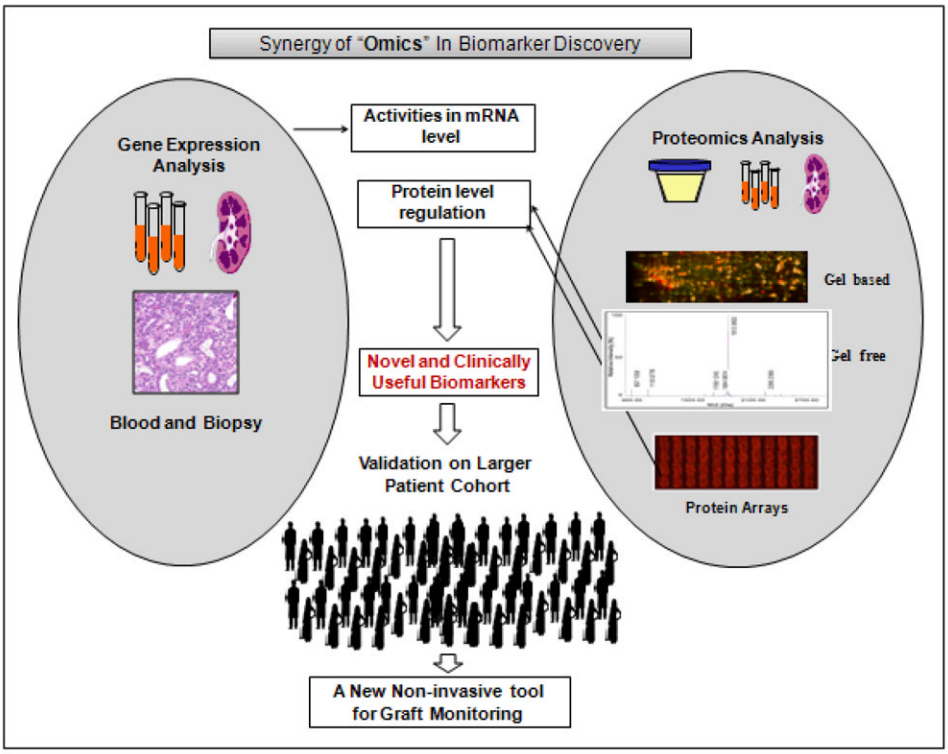

Figure 4.

A synergy by the integration of data available through different platforms and validation of these biomarkers using a larger cohort will be critical in reaching to a clinically useful biomarker in renal transplantation.

Table 1

Published studies using proteomics in renal transplantation in the past 9 years.

Methods	References	Number of Studied Patients	Sample Strategy
Gel-based method			
2D-DIGE/MS	Gao et al [80]	12	Pooled
	Banon-Maeus et al[40]	24	Pooled
Gel-free method			
SELDI-TOF MS	Clarke et al [81]	32	Individual
	Schaub et al [16]	50	Individual
	O'Riordan et al [82]	65	Individual
	Jahnukainen et al [36]	78	Individual
	O'Riordan et al[83]	45	Individual
MALDI-TOF MS	Quintana et al [84]	50	Individual
LC-MS/MS	Kurian et al [85]	9	Pooled
	Sidgel et al [65]	40	Pooled
	Freue et al [86]	33	Individual
	Nakorchevsky et al [68]	42	Pooled
	Wu et al [87]	18	Pooled
CE-MS	Wittke et al [88]	58	Individual
Peptidomics	Perez et al [89]	29	Individual
	Quintana et al [29]	32	Pooled/ Individual
	Ling et al [21]	70	Individual
	Sui et al [90]	36	Pooled
Protein microarrays method			
Protoarray	Li et al [41]	18	Individual
	Sutherland et al [43]	15	Individual
	Li et al [91]	22	Individual