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Interpreting gene-expression profiles in transplantation: a critical appraisal

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Oligonucleotide microarray technology has created a small revolution in the transplant community because it has helped to decipher previously unknown molecular processes involved in allograft pathology, redefined molecular patterns of diseases that are indistinguishable at the pathological level and made possible the definition of new prognostic factors for long-term graft outcomes. However, given the tremendous complexity of the biological processes that are involved in the pathology of a transplanted organ, the interpretation of transcriptomic data can be speculative and oversimplified. Here, we discuss critical considerations regarding the nature of the object studied by cDNA microarray technology, the means by which it is observed, the interpretation of the observations, and whether the observations make sense in the context of transplant-related scientific questions. Given these limitations, we believe that global approaches based on more functional biological intermediates are necessary for a better understanding of the molecular processes that regulate the physiopathology of the transplanted organ.

Keywords: kidney transplantation, cDNA microarrays, gene expression profiles

INTRODUCTION

Kidney allograft damage is often diagnosed too late, after irreversible tissue injury has been definitively established. A better molecular characterization of allograft injuries is needed to better prevent tissue remodeling. The reversibility of a lesion, independent of its origin, is closely related to early diagnosis, and such a diagnosis depends on the ability to detect changes in the inner workings of cells, i.e., at a molecular level.

Oligonucleotide microarray technology has had a tremendous success and has become a standard tool in molecular biology. This technology has created a small revolution in the transplant community. The application of microarrays to transplantation research has revealed previously unknown molecular processes involved in allograft pathology, including tissue responses to immunosuppressive drugs (Pallet et al., 2008; Dell'Oglio et al., 2010); redefined molecular disease patterns that are indistinguishable at the pathological level due to the heterogeneity of allograft rejection (Sarwal et al., 2003); and made possible the definition of new prognostic factors for long-term graft outcomes (Einecke et al., 2010). However, to date, these functional genomic tools have played an undefined role in clinical kidney transplantation diagnostics.

Transcriptomic analysis using oligonucleotide microarray technology relies on the observation of the state of a biological process, namely transcriptional regulation of genetic information, at a given time (the transcriptome). Technological platforms (the microarrays) generate huge amounts of data that are managed by sophisticated bioinformatics tools. From this basic description, elementary questions arise: What is the object we are studying? How do we observe it? How do we interpret our observations? Do our observations make sense?

These simple questions are important because interpreting transcriptomic data without considering the real functional significance of the mRNA transcripts (which are biological intermediates and not directly involved in functional cellular processes) is inherently speculative and oversimplified given the tremendous complexity of the biological processes that are involved in the pathology of a transplanted organ. We believe that global approaches based on more functional biological intermediates are necessary to better understand the molecular processes that regulate the physiopathology of a transplanted organ.

FUNCTIONAL INTERPRETATION OF mRNA EXPRESSION

THE INFORMATIVE VALUE OF mRNA

The biological process examined by oligonucleotide microarray technology is mRNA expression. mRNAs carry genomic information that will eventually be translated into a protein, whose function will depend on complex post-translational modifications. Consequently, the simple equation, "mRNA = function," e.g., transforming growth factor β (TGF-β) mRNA overexpression means "fibrosis," is probably overly speculative. A biological function results from the integration of numerous contradictory processes that constitute the "interactome," and therefore, the expression level of an mRNA is not necessarily informative of an ongoing biological process.

The overexpression of a given mRNA or cluster of mRNAs may be interpreted as an indication of the presence of a specific type of cell if the mRNA or cluster of mRNAs are assumed to be representative of the cell type. However, cells often express genes that are not usually associated with their lineage, e.g., epithelial cells express a wide array of proinflammatory (Bonventre and Zuk, 2004) and mesenchymal genes (Hertig et al., 2006), and immune cells express endothelial markers (Ma et al., 2010). Conversely, not all cells from the same lineage in the biological milieu express a so called "cell-type marker" (Zeisberg and Duffield, 2010). The great difficulty in defining specific cell markers at the protein level also applies to mRNAs, and one must be careful when interpreting the expression of a transcript as an indication of the presence of a given cell type.

EXPRESSION KINETICS

Transcriptomic analysis provides a global picture of a system at a given time and does not reflect the notion of flux, even if a short time course is performed. Flux refers to the integration of the production, accumulation and degradation of mRNAs. Thus, a given expression level *per se* is not indicative of the ongoing process that results in an increase or decrease in the transcript's expression. The mRNA expression level does not reveal the nature of the process driving the increase or decrease in mRNA expression. For example, mRNAs are degraded during cellular stress by mRNA decay (Hollien et al., 2009). This process affects mRNA expression level at a given time in the same manner as a repressor that affects the transcription rate. However, the biological meaning and implications of mRNA decay and repressor activation are fundamentally different and are not captured by a single-shot microarray analysis.

Emerging techniques address kinetics of RNA production, whose application in transplant-related diseases could provide additional information on pathophysiological processes not monitored by conventional transcriptomic analysis. These include the use of transcripts from nuclei (Barthelson et al., 2007; Zhang et al., 2008; Underwood et al., 2010; Yunger et al., 2010), which, for example, allow the visualization and analysis of mammalian mRNA transcriptional kinetics of single alleles in real time, or allow to simultaneously mapping single-stranded RNA regions in multiple non-coding RNAs with known structure. Another emerging method for the assessment of RNA kinetics is the dynamic transcriptome analysis (DTA). DTA is a non-perturbing method to measure mRNA synthesis and decay rates (Miller et al., 2011; Rabani et al., 2011). DTA involves genetically facilitated cellular uptake of the nucleoside analogs, metabolic RNA labeling, separation, and microarray analysis of RNA fractions. This is exemplified by analysis of the osmotic stress response, a conserved stress response pathway and one of the best-studied gene-regulatory systems in yeast (Miller et al., 2011).

NON-TRANSCRIPTIONALLY REGULATED BIOLOGICAL PROCESSES

Although each mRNA is translated into a protein at the basal level, transcriptional regulation represents just one of the many processes involved in the cellular response to environmental changes. Many fundamental biological processes implicated in transplantation are not transcriptionally regulated, meaning that they will not be detected by mRNA expression analysis.

Protein activation catalytic cascades, such as complement, coagulation, and caspase activation, are not regulated at the transcriptional level. For example, the transcriptome does not reflect the activation of complement during thrombotic microangiopathies or humoral rejection. Cell death processes (i.e., necrosis, apoptosis, and autophagy), which are present in an engrafted organ, (Galluzzi et al., 2012) may not involve the active expression of genes. Indeed, the effective activation and the execution of programmed cell death involve catalytic reactions (e.g., caspases activation during apoptosis), vesicle trafficking and fusion (in the case of autophagy), or signal transduction, kinases activation, and membrane permeabilization in the case of necrosis. Consequently, oligonucleotide microarray is not a useful tool to monitor cell death because there is no gene or cluster of genes whose expression is specifically attributed to a type of programmed cell death (Klionsky et al., 2008; Kepp et al., 2011). However, the basal expression of some genes encoding important regulators of programmed cell death is essential for the correct execution of programmed cell death sub-routines. This is exemplified by the dramatic phenotypic consequences of small RNA interference screens (Hitomi et al., 2008).

Transcriptional activity is not a key regulator of fibrogenesis. In addition to transcription, a central step in the induction of TGF-β is its activation from a reservoir of latent protein complexes in the extracellular matrix (Schnaper et al., 2009). TGF-β is basally secreted in the microenvironment and aggregates in its latent form with proteins in the extracellular matrix. Another important contributor to tissue fibrosis, connective tissue growth factor (CTGF), is released in a caspase 3-dependent manner independent of transcription (Laplante et al., 2010).

Non-conventional intercellular communications through membrane vesicles, which play a role in the modulation of alloimmunity (Thery et al., 2009), are non-transcriptional processes. Other fundamental biological processes that escape transcriptional regulation include protein degradation pathways (e.g., autophagy and the proteasome); kinase cascades; and the phosphoproteome; the ubiquitin system, which regulates HIF1-α and NF-κB signaling; mTOR and the integrated stress response that regulates transductional activity in response to ischemia (Bando et al., 2004); and intracellular vacuolar trafficking, which leads to MHC-peptide maturation and presentation.

In conclusion, this list of examples highlights the fact that the transcriptome represents only the tip of the iceberg and cannot detect a large number of fundamentally important biological pathways. Thus, reducing the tissular and cellular response to injury to the expression of transcripts can lead to biased and oversimplified conclusions.

Emerging methods could help to monitor mRNA actively translated in the cell (Zanetti et al., 2005; Halbeisen and Gerber, 2009; Halbeisen et al., 2009; Mustroph et al., 2009). In this regard, the translatome refers to the pool of all RNAs that are associated with ribosomes purified via an affinity tag that can be subsequently analyzed by microarray, and further compared with the transcriptome. As an example, the comparison of the relative changes of translatome and global transcript levels in response to stress that arrest cell growth correlate well with those in the translatome. However, this correlation is generally lost under more mild stresses that do not affect cell growth (Halbeisen and Gerber, 2009).

METHODOLOGY

Cultured cells are the simplest system for interpreting transcriptomic analysis because each transcript comes from the same cell lineage. However, this system is oversimplified because it lacks interactions with the surrounding microenvironment and intercellular communications. Furthermore, even in the case of a monotype cell culture system with a minimal number of interfering variables, reproducibility between experiments can be challenging. Such a simple model gives rise to complex and speculative interpretations about the consequences of the expression of the transcript of interest.

Conversely, transcriptomic analyses based on tissue extracts, such as kidney biopsies, integrate the transcript expression levels from many cell types, such as epithelial cells, endothelial cells, fibroblasts, and macrophages. It is clear that the transcripts present in a kidney biopsy are not produced uniformly by the organ as a whole but are a product of the cells within. As a result, the level of expression of the transcript τ represents the mean of all the expression levels (positive or negative) of τ in all cell types present in the biopsy. Thus, transcriptomic analysis of tissues reduces a multidimensional system (i.e., a multicellular and multicompartmental system) into an oversimplified, one-dimensional system with a tremendous loss of information (Shen-Orr et al., 2010). Because the expression level of a transcript integrates all cell types, it is difficult to ascertain whether its overexpression is a consequence of activation or of the accumulation of a particular cell type.

Overcoming these limitations remains a complicated task. Because the focal nature of structural alterations are common in the renal parenchyma in many human diseases and animal models, experimental methods for isolating subsets of tissues, such as cell sorting, enrichment, or the use of laser capture microdissected material, could be useful. However, these methods are prohibitively expensive and may affect cell physiology and gene-expression. Interestingly, flow cytometry and cell sorting can be employed for sorting nuclei form cells, that could allow to distinguish divergent cell populations within single biopsies that could help guide diagnoses and tailor approaches to personalized treatment (Ruiz et al., 2011). One solution could be the use of algorithms, such as cell-type specific significance analysis of microarrays (csSAM), that address the extensive loss of biological information in microarray datasets when analyzing complex tissue samples that vary in cellular composition. Such method can retrieve the information lost during microarray processing and lead to the discovery of significant genes that were otherwise undetectable (Shen-Orr et al., 2010).

RETHINKING TRANSCRIPTOMIC ANALYSIS IN TRANSPLANTATION

The tremendous success of transcriptomic analysis has relied on the relative simplicity of using the platforms, the small amounts of biological materials required, and the hope of discovering biological phenomena that were previously unknown. Moreover, sophisticated bioinformatics tools render the use of microarrays even simpler and more impressive by producing unsupervised hierarchical clusters, which can elucidate a previously unidentified molecular reality. These comprehensive analysis tools have been attractive because they may have generated the hope of revealing many mechanisms of transplant-related diseases without having to speculate. But in retrospect, it is possible that we have tended to overestimate their actual contribution to the understanding of disease processes in transplantation.

There are two major applications of transcriptomic data analysis. The first involves the definition of gene-expression profiles that can define a molecular signature of tissular injuries and that define subgroups of clinical situations that are undetectable by standard tools. As previously noted, such alterations of the expression of transcript clusters represent only a small component of the whole biological response to injury. Therefore, by using this application, we risk overestimating the informational value of studying transcription alone, which is partially disconnected from the final function we tend to assign.

The most exciting application of microarray analysis is to help identify novel biological pathways involved in a particular pathological or physiological setting, which must then be validated by cellular and molecular biology studies. These kinds of global approaches in the absence of an *a priori* hypothesis should raise additional questions and hypotheses. Such studies constitute the first step of mechanistic studies leading to a better understanding of the biological processes in which transcripts detected by transcriptomic studies may be implicated. Further characterization of such processes will lead to the definition of novel biomarkers and potential therapeutic targets. Because mRNAs are biological intermediates, one should not overestimate their significance, especially in cases of "data mining."

Transcriptomic profiling is being replaced by the applications of next generation sequencing (NGS). Applications of NGS in transplantation have not been published yet. NGS technologies constitute various strategies that rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods (Metzker, 2010). The increasing feasibility of whole genome and whole-exome sequencing (Choi et al., 2009; Cirulli et al., 2010), due to increased speed of "next generation" sequencers, and decreased cost may provide powerful tools to increase our understanding of the genome in transplant outcomes (Werner, 2010). For example, gene-expression studies microarrays are now being replaced by seq-based methods, which can identify and quantify rare transcripts without prior knowledge of a particular gene and can provide information regarding alternative splicing and sequence variation in identified genes (Ozsolak and Milos, 2011).

Rethinking global approaches to studying kidney transplantation involves redefining the analyzed biological target. Clearly, proteins are more closely linked to biological function, and integrated views of the proteome will potentially provide a more precise picture of tissue responses to injury. Recent technological progress in proteomics renders this approach attractive for fundamental scientific research on transplantation and also for molecular diagnosis. Nanoscale proteomics (Saba et al., 2009), subcellular and organelle proteomics (Boulais et al., 2010), proteomics of post-translational modifications, phosphorylation (Eyrich et al., 2011), the immunopeptidome (de Verteuil et al., 2010), and the antibodiome (Li et al., 2009) are examples of powerful technologies that could transform our understanding of kidney allografts as biologically integrated systems.

The tremendous progress in proteomic technology, particularly in mass spectrometry instrumentation, allowed proteomics to emerge as a powerful tool for biomarker research (Findeisen and

Neumaier, 2009). One advantage of proteomics is that it may analysis all the proteins in any defined biologic compartment, including urine, in a non-invasive manner. Whereas mRNAs can be isolated from urine (Muthukumar et al., 2005), the collection of adequate quality, and quantity material remains challenging. Proteomics may complements transcriptomic approaches because the proteome is the result of alternative splicing of primary transcripts, the presence of sequence polymorphisms, and post-translational modifications, which are not monitored by transcriptomic analyses. Additionally, there is no strict linear relationship between the genome and the proteome and often a poor correlation between mRNA abundance in a cell or tissue and the quantity of the corresponding functional protein. However, this may represents a challenging task as proteomes have a large and unknown complexity.

Before proteomics emerges as a reliable clinical diagnostic tool, some hurdles will have to be crossed, like the inconsistency in sample handling and processing and the lack of standardization of experimental design. Furthermore, robust study design with appropriate statistical power, blinding, and validation is of crucial importance to improve reliability of proteomic-driven results.

CONCLUSION

The arrival of "omics" in translational medicine, including transplant medicine, generated a conceptual twist in the formulation of scientific problems and the methods available to solve them. The classical approach is based on the formation of a hypothesis, followed by the search for an adequate model to test it through planned experiments that are performed step by step. The power of the new biotechnological tools, however, has led to an inversion of this process; the tool is first used to generate data, the data is interpreted, and finally a hypothesis is formulated. This is another way of doing science, and although it is a powerful and effective one, it should not overshadow its own limitations. A critical interpretation of gene profiling studies is always necessary.

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