At the Dawn of the Transcriptomic Medicine

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Running Title

Transcriptomic Medicine

Abstract

Progress in genomic analytical technologies has improved our possibilities to obtain information regarding DNA, RNA and their dynamic changes that occur over time or in response to specific challenges. This information describes the blueprint for cells, tissues and organisms and has fundamental importance for all living organisms. This review focuses on the technological challenges to analyse the transcriptome and what is the impact of transcriptomics on precision medicine. The transcriptome is a term that covers all RNA present in cells and a substantial part of it will never be translated into protein but is nevertheless functional in determining cell phenotype. Recent developments in transcriptomics have challenged the fundamentals of the central dogma of biology by providing evidence of pervasive transcription of the genome. Such massive transcriptional activity is challenging the definition of a gene and especially the term “pseudogene” that has now been demonstrated in many examples to be both transcribed and translated. We also review the common sources of biomaterials for transcriptomics and justify the suitability of whole blood RNA as the current optimal analyte for clinical transcriptomics. At the end of the review, a brief overview of the clinical implications of transcriptomics in clinical trial design and clinical diagnosis is given. Finally, we introduce the transcriptome as a target for modern drug development as a tool for extending our capacity for precision medicine in multiple diseases.

Keywords

Transcriptome, RNA-Seq, Gene Expression Profiling, Precision Medicine, Genomics, Molecular Targeted Therapy

Impact statement

This review describes the impact of transcriptomics on experimental biology and its integration into medical practice. Transcriptomics is an essential part of modern biomedical research based on highly sophisticated and reliable technology. Transcriptomics can aid clinical practice and improve the precision of clinical diagnoses and decision-making by complementing existing clinical best practice. The power of which will be increased when combined with genomic variation from genome wide association studies and next generation sequencing. We are witnessing the implementation of RNA-based technologies in clinical practice that will eventually lead to the establishment of transcriptional medicine as a routine tool in diagnosis.

Introduction

Since the identification of the structure of proteins and nucleic acids and the mechanisms of gene expression, the central concept of biology has underpinned our understanding of gene function[1]. According to this concept, the information in the cell is from DNA to RNA and subsequently translated into proteins. Therefore, the function of genes should be analysed only by their ability to produce proteins and that proteins define phenotype. One field of research, transcriptomics, has revolutionised this central biological concept. Discovery of the abundance and complexity of RNA dynamics and function dramatically changed our understanding about the role of RNA, apart from encoding proteins, and challenged gene-centric approach to explain the function of genome[2]. Transcriptome is a collective term describing all RNAs produced by a single cell, by a population of cells or tissue[3]. Recent progress in analytical technologies has unveiled the complexity of the regulation of the transcriptome. The transcriptome is the primary product of the genome and therefore analysis of the transcriptome provides primary information for functional genomics.

The human genome

One of original and the most remarkable results of the human genome project was the discovery that only 1.2% of the human genome encodes proteins and was therefore considered as functional and meaningful [4]. This is also reflected in the early search for genetic variation associated with a specific disease focusing on DNA sequences solely in exons. The number of genes, protein-coding elements, was discovered to be around 30,000, a much smaller number than predicted and similar to that found in several other species [4]. Based on these findings the rest of the genome was initially termed as junk DNA. However, additional studies have identified that most of the DNA has function, not only for genome

structure and packaging, but also to form the complexity of the molecular networks underpinning the diversity of cell function. Early studies, after the identification of individual chromosome sequences, indicated that genomic sequences were transcribed at least as much as an order of magnitude more than accounted for by the predicted gene models[2]. Similarly, the term "pseudogene" that implies that is not a real gene and considered as a remnant of evolution or "genomic fossil"[5]. It is now demonstrated that most of the pseudogenes are transcribed and translated into proteins challenging that definition of "pseudogene"[6]. Cap-analysis gene expression (CAGE) technology enabled the identification of at least 180,000 transcripts in the mammalian genome and it appeared that the majority of the genome is transcribed [7]. At least 60% of the genome has been described as a transcriptional forest, where transcription is performed from both strands of the same DNA region without gaps [7]. The most remarkable project in this field is known as an Encyclopedia of DNA Elements or ENCODE for short. Based on ENCODE findings at least 80% of genome is actively transcribed and this number is considered to be conservative[8]. Such data requires we review our interpretation of genome function and regulation and how that is utilised in clinical translation.

Transcriptome, transcriptomics and transcriptome profiling

Transcriptome is a collection of the RNAs (transcripts) that single cell or tissue can produce, and it contains all types of RNAs[9]. Transcriptomics is the study of the transcriptome; analysing RNA and its different subcategories (mRNA, micro-RNA, non-coding RNA, etc) to identify changes in expression and its functional impact. Although transcriptomics focusses on content and transcript expression levels, it also includes the analysis of transcriptional regulation. The transcriptome can be studied by different methods, however the most

common options are genechips (to measure gene expression on microarray platform) and RNA sequencing (RNA-seq) [9]. Gene expression arrays initially focused solely on polyA purified RNA that encode proteins. Moreover, genechips also suffer from the requirement to be pre-designed, i.e. the content on the array is based on our pre-existing knowledge of predominantly exons that can be easily identified in genome sequence data [10, 11]. Therefore, genechips give us a snapshot of the transcriptional changes of mRNA, but this snapshot is rather limited. More recent arrays (transcript based and tiling arrays) can give very comprehensive information about the transcriptional changes, nevertheless the genechips are inherently bound to pre-existing knowledge and do not provide information about the sequences of the transcripts [12,10]. Only a few genechip versions are capable of identifying alternative splicing and specialised chip design is required to analyse such as micro-RNAs[12]. But the sequence information is lost in results files, and this is where the RNA-sequencing has clear advantage allowing for more detailed analysis to detect alternative splicing, intron retention and other events reflecting alterations in transcriptome regulation and the other classes of RNA. Therefore, RNA-sequencing has become the main technology for transcriptome analysis[9, 13].

Sources of the transcriptome

Gene expression is both tissue specific and stimulus inducible therefore a key question for transcriptome analysis is the source of the tissue or cell type for analysis. The most common and easiest to justify is the primary tissue that is affected by pathological processes. This is based on the assumption that we know what tissue is affected and we have some preliminary understanding what the timeline and mechanisms of the pathological changes are. However, this assumption can be deceiving. For example, with CNS disorders, it is

difficult to determine which region or cell type is involved and also whether the pathological hallmarks of the disease were initiated by dysfunction in another brain regions, or periphery, many years before. As brain tissue is only accessible as post-mortem tissue, the changes in the transcriptome could arise from selective alteration of gene expression by the post-mortem time rather than in response to living with a chronic age-dependent disease occurring over a long time period [14, 15,16]. In case of neurodegenerative diseases, this may mean that we miss the molecular pathological changes that initiate the degenerative process. The same is similar for other chronic age-dependent disease such as arthritis or heart disease. The cells that are targeted by primary pathology are often dead or have a significantly altered phenotype from those that represent the key pathological transitions. Some of the problems of addressing transcriptomics in the CNS are outlined below.

Firstly, recognised issues with the use of biobanked tissue samples that would affect transcriptomics include the heterogeneity of the samples, reliability of the diagnoses and variability in the quality control measures [17]. The most drastic example to illustrate reliability challenges comes from the biobank having 12,000 samples available for research and only 18 of them with the suitable information and quality by the end [16]. While the analysis of post-mortem brain samples is still valid and informative from a research point of view, the impact of these studies to improve our understanding about neurodegenerative disease needs addressed in a broader context [16]. It is difficult to infer causative changes from the single time point that is based on the analysis of the tissues where the pathogenic processes are completed.

Secondly, subjects may have used drugs for a long time and depending on the course of the disease the treatment schedules can be quite different between patients [17]. Moreover, it is quite realistic to assume that the subjects have had comorbidities and taken drugs for those

symptoms as well. Drugs for heart disease and hypertension and statins are quite common in the aged population and therefore analysis of the post-mortem samples should most certainly take into account the drug history and comorbidities as confounders. This is something we do not see very often in studies using post-mortem tissue samples.

Thirdly, we need to consider what regions of the tissue is to be analysed. Again, in the case of the brain, regional changes in gene expression can be enormous [17]. It is a complex tissue and choosing the right regions for comparison is often the most important decision for the analysis. For example, in the case of targeted mutation mouse models generated by homologous recombination the changes in the transcriptome of the brain are regionally very different [18-20]. Targeted mutant mouse lines allow exclusion all confounding factors and careful matching of the study subjects for the genetically engineered mutations. However, even after the perfect matching for confounders, the deletion of the single gene induced enormously different changes in transcriptome in the different regions of the brain [20]. Only the lack of the expression of the deleted gene was the similar result between the different brain regions [20]. In addition to the regional difference in the brain tissue, genomic locus of the gene has also to be considered. We have analysed the transcriptome of the Wolfram syndrome mutant mice with the deletion of the Wfs1 gene and identified significant confounding effect from the genomic locus of the targeted gene [18]. This locus-specific or genomic context effect means that even a single gene targeting, or deletion can induce the complex changes in the transcriptome that are not caused by the function of the gene, but by its location. Mouse models enable controlling for gender, age and environmental differences, providing the ideal study design conditions, but cannot avoid genomic background effect, “congenic footprint” [21]. This effect needs to be taken into account and with appropriate adjustment the functionally meaningful differences can be identified [22]. All

this illustrates how diverse the transcriptome is in different brain regions and therefore it is challenging to design studies with multiple brain regions involved as it is not trivial to differentiate between the normal regional and pathologically relevant differences. In summary, by analysing post-mortem brains we struggle to obtain the relevant information about the mechanisms of the disease and this information does not always help us to design better diagnostic tools or drugs.

However, analysis of the diseased tissues is important when it is possible during the pathogenesis of the disease. Repeated sampling during the course of the disease allows us to use the time-dependent causative interaction models. Longitudinal studies are therefore the best way to follow disease progression but severely limit the choice of tissue or component that can be measured to such as blood, skin, urine and microbiome. This also enables the monitoring of changes in the transcriptome during treatment and to compare different therapeutic options [23]. In more limited cases, surgical removal of tissue during medical procedures is another option to access samples for transcriptomic analysis. The latter option is the most common for oncological samples and is potentially applicable for any surgically treated conditions. If we plan to perform longitudinal transcriptome analysis with samples from different time-points, then almost the only viable option is blood sampling. Skin sampling can also be alternative for some cases and diagnoses. We have shown that skin and blood are useful alternatives even for neurodegenerative diseases like Parkinson's disease [24-26]. Both blood and skin showed clear transcriptome differences in the case-control design and these tissues could be used for the diagnosis or monitoring the progression of the disease. Similarly, urine can be used as a source for transcriptome analysis [27, 28]. However, as usually the cellular content in urine is low, the RNA level is also

low and that reduces potential of urine or other body fluids as a source for transcriptomics [28].

Whole blood versus PBMC transcriptome

Blood is a useful and easy to access surrogate tissue for transcriptome analysis, but the use of blood requires a few basic decisions. For example, it is possible to analyse whole blood or a particular fraction of blood cells. Peripheral Blood Mononuclear Cell (PBMC) separation has been one very popular method to isolate cells from the blood and to prepare them for RNA analysis. However, the PBMC fraction contains only lymphocytes and monocytes while all granulocytes like basophils, eosinophils and neutrophils are depleted. From all white cell count, neutrophils constitute 55 to 75% indicating that using of PBMC for transcriptome analysis would not give the full picture [29]. Isolation of PBMCs covers only 20 to 50% of the cellular heterogeneity of the blood. Moreover, PBMC separation itself is a procedure that adds an extra uncontrollable variation to the analysis, and this should be avoided. Several studies have shown significant differences between the transcriptome profiles between PBMC and whole blood [30]. It is reported that over 2,000 genes were differentially expressed with more than 2-fold difference between PBMC and whole blood from the same individual at same time [31]. Therefore, for transcriptome analysis the whole blood RNA samples have a substantial advantage over PBMC or other fractionation.

Preanalytical considerations

Due to the complexity and the volume of the transcriptomics data preanalytical conditions have significant impact on the outcome of the analysis. The inadvertent variations can be introduced with the sampling of the tissue, during the storage and transportation or by the differences in the extraction methods. In addition, as addressed in previous sections, the

sources for RNA can be variable ranging from blood and other body fluids to the tissue biopsies, cellular smears and to single cell sorting. All these different approaches require standardised protocols to ensure reproducibility and high quality of the analysis. The testing and guidelines how to prepare and purify different clinical samples is vital for the further implementation of the transcriptomic analysis in clinical practise. RNA extraction can be notoriously complicated with variable options available that all can lead to different results [32]. Similarly, storage conditions have been shown to impact the quality of RNA and snap-frozen samples detect signifficantly more genes than FFPE samples [33]. This effect was not dependent on the time to fixation. Interestingly, miRNA expression was not affected by the fixation method and it was comparable between frozen or FFPE samples [33]. In addition, purification of the liquid biopsy samples requires an extra effort and a complex workflow [34]. As RNA can be purified from different samples, validation studies are required to develop standardised protocols that would enable robust and reproducible analysis of transcriptome for various clinical conditions.

Practical utility of transcriptome analysis

The transcriptome is a snapshot of molecular events in the cell reflecting the functional activity of the genome at a given moment of time and requires a combination of analytical tools to describe these molecular changes. Currently, the majority of genomic tools used in clinical genomics only consider targeted DNA sequencing and not the transcriptome. However, there are several examples of how transcriptomic information improves the precision of the genomic analysis.

The early studies to analyse transcriptomics used variable differential cloning technologies based on cDNA library preparation and comparative analysis [35]. One of these methods, cDNA Representational Difference Analysis (cDNA-RDA), was used to identify differential

expression in pancreatic cancer [36]. cDNA-RDA was proven to be a highly efficient and reproducible method that has been used in various models and organisms [37, 38]. While the method itself was laborious and difficult to use for larger sample numbers, it clearly had its advantage as a hypothesis-free approach to observe transcriptional changes [39]. As the method did not require specific equipment or expensive preparations like gene microchips, the method gained popularity and was applied to study variable pathologies or physiological responses [40]. At the same time cDNA microarray technology was also developing and provided various in-house products These microarrays were based on the cDNA clone collections, their amplification and printing (spotting) on to glass slides [41]. This technology required substantial infrastructure to run and it wasn't widely accessible. Nevertheless, the initial studies demonstrated their suitability for pathology and clinical diagnostics in particular in cancer were tumour material was available. These studies indicated that breast cancers can be classified by their gene expression patterns into subtypes that were not identifiable with histological methods alone [42]. The gene expression pattern was not only helpful to identify the molecular subtypes of the breast cancers, but also to predict the clinical course and outcomes of breast cancer [43]. These early reports fuelled a myriad of similar studies to determine the transcriptional pattern of other tumours to identify potential diagnostic or prognostic biomarkers. Gene microarrays became standardised for transcriptional studies. The main advantage was the high-throughput analysis of the cDNA libraries and as the technology was scalable it was possible to increase sample sizes and the power of studies. However, gene arrays still suffered from a biased capture of targets which as stated previously were based on exon data or a limited number of non-coding RNAs. The latter was partially resolved when Next Generation sequencing (NGS) technologies become easily accessible to enable parallel whole genome sequencing (WGS) and RNA-sequencing

(RNA-seq). Clinical genetics analysis rapidly expanded from exome sequences to a complete RNA analysis. RNA-seq is the first technology that enabled complete transcriptome analysis covering all different types of RNA subclasses with complete sequence information and enables detection of complex profiles from various pathologies [9].

Several examples support the value and the utility of transcriptomics in the complex analysis of clinical samples for association to disease. We have analysed the transcriptional profiles of osteosarcoma samples from fresh tumours in a paired study design and identified several new candidates involved in the development of osteosarcoma[23]. Moreover, with similar technology we were able to analyse archived formalin fixed paraffin embedded (FFPE) samples that gave us the possibility to evaluate the effect of chemotherapy on the transcriptional profile. The same dataset provided data regarding repetitive elements that were differentially expressed in the malignancy [44]. Repetitive elements can only be efficiently analysed using the RNA-seq technology rather than genechips.

Transcriptome analysis can stratify patients who would otherwise be grouped as the same disease and this enables biomarker-driven clinical trials to improve their efficacy. Several meta-analyses have shown substantial improvement in study outcomes by using the biomarker-driven stratification in the study designs [45]. Personalised medicine approaches involving biomarkers in study design improved response rates from 5 to 30 percent demonstrating the improvement that can be achieved by using a genomics driven approach[45]. For example, the Winther trial based on 303 patients utilised genomic-matching to personalise their cancer therapy [46]. The study had two arms, one was based only on DNA data and the other only on RNA data. This trial introduced several innovative paradigm shifts showing an improved therapeutic response with the integration of transcriptomic profiling. Most importantly, the transcriptomic arm identified the most suitable solutions for

the patients with various solid tumours prospectively from the large database of therapies [46]. This trial considered patient therapy options at an individual level based on the features of person's tumour and not on the results obtained from the aggregation of trials on large patient populations. Therapeutic guidance based only on the transcriptomic data resulted in the stabilising of disease in 30% of patients [46]. While not statistically superior from the DNA-only approach (26%), it is was a remarkable success considering that the study subjects all had advanced cancers with several previous therapies that were unsuccessful.

Transcriptomic-guided therapy was considered because the DNA analysis alone does not often reveal actionable variants or mutations and RNA analysis could indicate the functional consequences. RNA-sequencing served here as an additional analytical tool to describe the functional changes in cancer that was in turn used in the therapeutic decision pipeline.

NGS technologies have also changed the ways we analyse Mendelian diseases and made whole-exome sequencing (WES) or WGS accessible to identify disease-causing variants. However, the success rate for detecting causal changes ranges only from 20 to 30% [47]. In a recent study, the use of RNA-seq analysis yielded diagnostic rate of 35% on previously unsolved cases by WGS analysis indicating a marked improvement [48]. The main advantage of RNA-seq is its ability to detect aberrant splicing or disruptive changes in the transcriptional regulation that are not detectable with WGS or WES [48]. This is the evidence to support the power of RNA-seq analysis also for Mendelian diseases and shows its clinical applicability in this space.

A recent example for the applicability of transcriptome analysis or RNA-based diagnostics can be found from the COVID-19 pandemic caused by the RNA-virus SARS-CoV2. The virus is only 29,900 bp long and contains 10 genes with gene 5 and 7 being functionally bicistronic [49]. Infection is based on the infectious transcriptome and can be viewed as a transcriptome

infection. Maybe the efficient therapy for viral infections lies in the targeting of the transcriptome to affect their transcriptional capacity. Transcriptome based therapies are already available for human diseases like Duchenne Muscular Dystrophy or amyloidosis showing the potential of the transcriptome based therapeutics [50-52]. Transcriptome-based therapies offer a real systematic opportunity for personalized medicine and it requires complex transcriptome analysis as input [53]. This therapeutic approach can turn the information in the transcriptomics into therapeutic options.

Conclusion

Transcriptomics is currently a rapidly evolving field with new data to either stand alone or integrate with other clinical information to expand and modify the future of health care. While current applications are mostly limited to experimental projects, a growing number of studies indicate the practical utility of transcriptomics for diagnostics, genomics-driven trial design and personalised drug development. Larger clinical validation of such experimental hypothesis will allow for accepted clinical usage, indeed blood samples can be taken in general practice and sent off for analysis and interpretation centrally before transmission to the clinician. Transcriptomics has revealed the vast complexity of the transcriptome and we are just beginning to understand the principles of how this translates to function, pathophysiolgy and therapeutic opportunities.

Authors' Contributions:

GK, ALP, VJB, JPQ and SK conceived the idea, performed literature search, drafted manuscript and worked with the final version. All authors participated equally.

Declaration of Conflicting Interests

Authors declare that they do not have conflicting interests regarding to the subject of this manuscript.

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