Mini-Review • DOI: 10.2478/s13380-011-0005-6 • Translational Neuroscience • 2(1) • 2011 • 69-75

Translational Neuroscience

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QUEST FOR NEW GENOMIC AND PROTEOMIC BIOMARKERS IN NEUROLOGY

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Abstract

The possibility of identifying novel biomarkers for neurodegenerative diseases has been greatly enhanced with recent advances in genomics and proteomics. Novel technologies have the potential to hasten the development of new biomarkers useful as predictors of disease etiology and outcome, as well as responsiveness to therapy. Disease-modifying new therapies are very much needed in modern approaches to treatment of neurodegenerative diseases. Current progress in the field encounters a degree of skepticism about the reliability of genomic and proteomic data and its relevance for clinical applications. Standard operating procedures covering sample collection, methodology and statistical analysis need to be fully developed and strictly adhered to in order to assure reproducible and clinically relevant results. Previous studies involving patients with neurodegenerative diseases show promise in using genomic and proteomic approaches for development of new biomarkers. Confirmation of any novel biomarker in multiple independent patient cohorts and correlation of the improvement in biomarker endpoint with clinical improvement in longitudinal patient studies remains crucial for future successful application. We propose that a combination of approaches in biomarker discovery may in the end lead to identification of promising candidates at DNA, RNA, protein and small molecule level.

Keywords

• Biomarkers • Neurology • Genomics • Proteomics

Received 24 February 2011
accepted 03 March 2011

1. Introduction

Biomarkers are biological traits which can be measured objectively in peripheral blood, cerebrospinal fluid (CSF) or other biological samples, as well as imaging procedures, and which can be used as indicators of normal or pathological biological processes [1]. Biomarker discovery studies offer clinicians and scientists a novel approach to treating and understanding diseases as they can be invaluable in determining the staging of a disease, classification of disease dissemination, prognosis of disease outcome and monitoring of the clinical treatment [2].

Neurodegenerative diseases are a clinically heterogeneous group of diseases marked by a progressive loss of neurons within the central nervous system resulting in pathological lesions and clinical manifestations ranging from movement disturbances to cognitive and psychiatric symptoms. Noninvasive biomarkers which could be used for diagnostics in certain neurodegenerative diseases, such as Parkinson's disease, or as tools for discovery of disease-modifying novel therapies are currently much needed [3]. Namely, biomarkers which could be used as surrogate-endpoints in clinical trials, enabling selection of most promising disease-modifying compounds, would prove invaluable [4]. Additionally, disease progression biomarkers would enable administration of appropriate therapy at the most opportune time, for instance early in the disease in asymptomatic carriers of mutation for Huntington's disease or spinocerebellar ataxias.

All putative biomarkers need to be validated in distinct cohorts of patients in prospective, multi-centric studies. Criteria for validation of a prospective biomarker need to be determined according to its role. Namely, a possible diagnostic biomarker might not be useful in monitoring response to treatment. Recent progress in the knowledge of the genetic and molecular etiology of neurodegenerative diseases has provided the basis to develop genomic and proteomic biomarkers that may be important in the development of new treatment strategies.

2. Novel genomic approaches in predictive medicine

Achievements of the Human Genome Project and the complete decoding of the human genome have opened an unimaginable set of opportunities for scientists to further unveil delicate mechanisms underlying the functional homeostasis of biological systems [5]. Microarrays or gene chips present a powerful tool for high-throughput analysis of transcriptome and have been used extensively in studies aimed at determining biological mechanisms involved in disease onset and progression both in tissues and bodily fluids [6]. The traditional approach of analyzing affected tissues derived from patients provides a useful insight into disease pathophysiology. However, affected tissues are not always easily accessible,

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as is the case in neurodegenerative diseases [7]. Peripheral blood and other bodily fluids, such as CSF, can provide an easily accessible substrate for microarray analysis aimed at biomarker discovery. One investigation, for example, reported that 80% of genes in the human genome are expressed by human peripheral blood cells [8] and it has been shown that peripheral blood cells share significant gene expression similarities with central nervous system (CNS) tissues [9]. Expression profiling of whole blood RNA offers understanding dynamic insight into aberrant patterns of gene regulation in neurodegenerative processes, allowing for detection of changes much earlier when compared to proteomic analysis. Transcriptome studies also have an advantage when compared to proteome and metabolome studies because of the uniform chemical nature of RNA.

Several neurodegenerative diseases elicit changes in peripheral blood cells which are specific for patients when compared to healthy controls [10-15]. Furthermore, even in diseases with no definite disease-associated phenotype in blood cells of patients, expression profiling studies have yielded disease-specific patterns which may be used for development of novel biomarkers [16-18]. More generally, there are numerous examples of the use of genomic technologies to identify novel biomarkers for several neurological diseases [19-24].

2.1 Technical aspects of blood expression profiling

Peripheral blood as a substrate for expression profiling experiments is subject to inherent variability, stemming from sample collection techniques (whole blood or peripheral blood mononuclear cell extraction), ex vivo handling of blood cells prior to RNA extraction, changes in blood cell count of individuals assayed, variations related to the stage of the disease (differences in disease symptomatology or disease activity) and changes in RNA expression of blood cells incurred by infection or drug administration [25]. Standardization of the patient selection and blood collection procedure is crucial in order to avoid these sources of variability as they may affect the final results of the study and reproducibility of the findings. Collection of whole blood using RNA stabilization reagents may provide a way to avoid variability caused by handling of blood cells. This has been solved by the availability of commercial tubes that stabilize RNA immediately upon collection. Standardization of blood collection time and adhering to strict timeframe from blood collection to blood processing are equally important [26]. A recent study identified many new candidate genes that are differentially expressed according to inter-individual (i.e. fasting, body mass index) and exposure (i.e. smoking) factors, establishing that these effects are also mirrored in blood [27]. Furthermore, assessing the blood cell count of patients, exclusion of patients with infection and keeping track of therapy the patient is receiving will also reduce the bias which can influence the final results of the studies.

2.2 Development of hemogenomic biomarkers in neurology and psychiatry

Several key limitations must be taken into account when performing expression profiling experiments using peripheral blood samples. The biological variability, namely intra-individual and inter-individual differences, always present a possible problem [28]. The best way to address the problem is to use a large number of samples, divided into a separate training set used to select the biomarker, and preferably several independent test sets, used to validate the biomarker.

Additionally, a certain degree of concern regarding technical variability is always present, particularly when different microarrays and manufacturer specific protocols are used as this may influence the final results of expression profiling experiments. Studies, such as Microarray Quality Control project have provided some reassurance about the reproducibility of contemporary microarray platforms, showing an average 89% overlap in expression profiles generated between sites using the same microarray platforms and 74% overlap across platforms from different manufacturers [29]. Furthermore, analysis of the samples using two or more microarray platforms and selecting the most reproducible differentially expressed genes as biomarkers, provides one way of reducing the influence of inter-platform technical variability in the biomarker selection process. More recently, wider accessibility of RNA sequencing approaches has provided a convenient way of circumventing the aforementioned technical variability.

Finally, proper selection of statistical methods used is crucial, as it can also be a source of bias in the procedure of new biomarker selection. Microarray studies generate a large amount of data which must be analyzed to show biological significance of the observed differential gene expression patterns [30]. Non-biological experimental variation or "batch effects" are commonly observed across multiple batches of microarray experiments, often making the task of combining data from these batches difficult. The ability to combine microarray data sets allows researchers to increase statistical power. Traditionally, biomarkers have been introduced into clinical practice based on discovery of their biological function within a specific disease. The major limitation of this approach is that our knowledge of disease mechanisms limits our ability of biomarker selection. Modern expression profiling experiments, especially in blood of patients with neurodegenerative disease, rely usually on a purely statistical approach of feature selection, according to significance of differential biomarker gene expression, regardless of their biological role. The aforementioned approach offers a better chance of discovering novel genes involved in disease pathophysiology. However, in some cases it may lead to selection of biomarker genes which do not reflect disease specific processes and which may later prove to be invalid. Combing the two described approaches by utilizing both the information on most significant differentially expressed genes and most significant enriched functional groups of genes relevant to disease pathophysiology, could improve the results in the process of biomarker discovery. In this way one does not only select the statistically valid biomarker set, but also takes into account general pattern of gene expression, which may reflect disease-specific processes.

Overall changes in gene expression are subtle, implying the need for careful analytic approaches to the data.

3. Proteomic approaches in biomarker discovery

Modern advances in proteomics as a study of both the strucutre and function of proteins, have radically improved the speed and precision of protein identification and quantification in biological fluids and other samples. Nevertheless, the sheer intricacy of biological systems and the complex nature of proteins, ranging from sequence perturbations, to conformational changes and post-translational modifications, pose a substantial hindrance to performing unbiased proteomics profiling. [31]. As in genomic approaches, several components of the analysis process have to be performed with utmost scrutiny, including sample preparation, protein or peptide separation, protein or peptide identification and bioinformatic data processing. Numerous AD, PD and HD studies have demonstrated significant promise of the proteomics profiling for selection of potential biomarkers [32-38].

3.1 Technical aspects of proteomics analysis

The array of target tissues which can be analyzed in patients with neurodegenerative diseases includes plasma, CSF and postmortem brain samples, as well as other peripheral fluids such as saliva. However, CSF, being most proximal to the brain structures undergoing degeneration, has been viewed as an ideal source of diagnostic, prognostic and therapeutic biomarkers. It is also considered as a relatively minimally invasive procedure that can be performed at any time during disease progression and repeated in the same individual [39]. Proteomic approaches in CSF analysis have already provided a degree of success in neurodegenerative diseases such as AD [40] and Creutzfeldt-Jakob disease [41]. Use of CSF as a substrate for proteomic analyses has provided many possible biomarker candidates for neurodegenerative diseases and studies carried out so far have identified more than 2500 proteins in human CSF [31,42,43]. Simple proteomic blood-based biomarkers would represent a great tool for early diagnosis and monitoring of disease progression. Although such approaches have been utilized, a conclusive proteomic biomarker from plasma of patients with neurodegenerative diseases still needs to be identified.

Analytical approaches also represent a source of possible technological variability. Proteomic analysis consists of two general steps: fractionation of the complex protein mixture and identification and quantification of the separated proteins. Fractionation is usually accomplished using 2-dimensional gel electrophoresis (2-DGE) [44,45], liquid chromatography (LC) [46], or more recently protein microarrays [47]. The fast-developing technologies of quantitative proteomics also provide a unique opportunity to reveal changes in a protein profile. Proteins within the simplified mixture are typically identified using a mass spectrometry (MS) based approach, which consists of three major modules, namely the ion source, mass analyzer and the detection unit [31]. Based on the difference in the ion source used, most of the mass spectrometers can be generally divided into electrospray ionization (ESI) or matrix assisted laser desorption/ionization (MALDI) instruments. Among the mass analyzers several are most widely used such as ion trap, triple quadrupole, time-of-flight (TOF) and Fourier transform ion cyclotron (FTICR). Surface enhanced laser desorption/ionization (SELDI) is basically a variation of the MALDI concept, which utilizes either a chemically prefabricated surface or a protein specific surface for selective capture of proteins [48]. All of the aforementioned instruments are different in their mechanisms of ion separation, mass accuracy and resolution, and complementarily in protein identification when used in concert [49]. Overall, these methods allow for high-throughput quantification of global protein expression in heterogeneous tissue samples and are therefore efficient tools in the search for neurological disease biomarkers. However, specific combinations of different modules may influence the final results of the analysis, prompting careful selection in methodological approaches utilized.

3.2 Avenues for development of novel proteomic biomarkers

Recent studies involving copy number variation analyses have indicated a possible role for increased copy number of specific genes and probably gene product levels in the pathophysiology of neurodegenerative diseases [50]. Furthermore, a necessary step towards clinical use of a potential biomarker is to detect quantitative alterations of protein levels under different disease and control settings. Thus, development of novel quantitative methods in proteomics remains a chief goal in novel biomarker development. Traditional approaches using 2-DGE have shown several limitations in that respect, namely it is labor-intensive, not applicable for proteins/peptides smaller than 10kDa, troubled by co-migration issues and has limited use for highly hydrophobic proteins [51,52]. On the other hand, MS-based quantitative methods have been refined in recent years, especially through development of isotopic tags at specific functional groups of peptides or proteins, among others isotope coded affinity tags (ICAT) [53] and isobaric tags for relative and absolute quantitation (iTRAQ) [54]. Additionally, label-free quantitative approaches using LC-MS/MS have been developing rapidly in recent years.

Post-translational protein modification has been examined more extensively of late, in an effort to elucidate its possible role in protein miss-folding, aggregation and degradation. These modifications include oxidation, nitration, S-nitrosylation, phosphorylation, ubiqitination and glycosylation. Stemming from the overwhelming evidence for oxidative or nitrative stress as key factors involved in neurodegeneration [55], several studies have shown the role of oxidative modifications or nitration of specific proteins in promoting protein aggregation [56,57]. These analyses rely mostly on detection of protein carbonyls, tyrosine nitration or cysteine S-nitrosylation. Protein phosphorylation is one of the most frequent post-translational modifications and a critical regulatory mechanism of cellular homeostasis influencing such diverse processes as proliferation, gene expression or signal transduction [58-60]. The role of phosphorylation has been well-evidenced in

neurodegenerative diseases, such as increase in the concentration of free hyperphosphorylated tau in AD or other tauopathies [39]. Covalent addition of single or multiple units of ubiquitin, typical to lysine residues, is a crucial mechanism involved in the targeting of intracellular proteins for 26S proteosomal degradation but also in numerous other functions [61]. Dysregulated protein degradation, both through the ubiquitine-proteasome and the lysosomal pathway, has been implicated in neurodegenerative diseases. The study of ubiquinated proteins provides a direct tool for assessment of the role of the ubiquitine-proteasome pathway in neurodegeneration. Additionally, altered proteolytic cleavage has been implicated in AD, where abnormal involvement of sequentially active secretases leads to accumulation of pathogenic A-beta 42, and in HD, where short N-terminal fragments of polyglutamine repeats seem to be more toxic than full-length huntingtin [62].

Finally, protein glycosylation has been emerging as an important source of protein diversity and a viable target for development of novel biomarkers [63]. There are four known categories of glycosylation, namely N-glycosylation, O-glycosylation, glycophosphatidylinositol anchors and C-glycosylation [64]. Aberrant glycosylation changes have been shown to occur in AD [65], both in the CSF and in the post-mortem AD patients brain samples. Additionally, Reelin, a glycoprotein that is essential for correct cytoarchitectonic organization in CNS was found to be upregulated in several neurodegenerative disorders, such as frontotemporal dementia, progressive supranuclear palsy PD and AD [66]. A more thorough pursuit of investigations into the role of protein modifications may provide additional insight into neurodegenerative processes and may yield novel targets for biomarker development.

4. Conclusion

The possibility of identifying novel biomarkers for neurodegenerative diseases has been greatly enhanced with recent advances in genomics and proteomics. In some cases, such biomarkers may prove invaluable in diagnostics of neurodegenerative diseases dependent on purely clinical diagnosis, such as PD. Additionally, in asymptomatic carriers of causative mutations such biomarkers could prove important in prediction of disease onset or monitoring of disease progression. To ensure predictive values of biomarkers in independent populations, the use of large number of individuals, in several independent patient cohorts is of great importance. In that respect, clinical introduction of any new genomic or proteomic biomarker will request procedure resembling a workflow of a large clinical trial.

More importantly, genomic and proteomic biomarkers could be most valuable in monitoring response to therapy, possibly ensuring a way to assess therapeutic efficacy. The efforts aimed at the search for new disease modifying therapies, which are very much needed in modern approaches to treatment of neurodegenerative diseases, could benefit most. Such biomarkers could serve as surrogate endpoints in clinical trials, enabling in vivo screening and selection of the most promising compounds. This approach would also allow for significant reduction in costs and time needed for testing of new disease modifying drugs. As drug development moves into the next decade, there are increasing expectations that medicine will be personalized with increased efficacy and reduced risks of adverse events. Mentioned advantages show that novel genomic and proteomic approaches have great potential to be widely used in drug discovery.

Extreme care has to be taken when selecting such surrogate endpoint markers to be sure that they really reflect disease pathophysiology, since selection or rejection of novel therapeutic agents based on genomic or proteomic biomarker could potentially lead to elimination of possibly efficient compounds. It is important to note that one biomarker gene set might not be able to perform all of the functions described above. It is feasible to predict that several biomarker sets might be needed for one disease in order to fulfill the functions of a diagnostic, prognostic and surrogate endpoint biomarker.

Whatever the possible function of the novel biomarker may be, strict protocols covering all aspects of methodology used, ranging from sample collection, RNA or protein extraction, analytic approaches to statistical workflow, will have to be developed in order

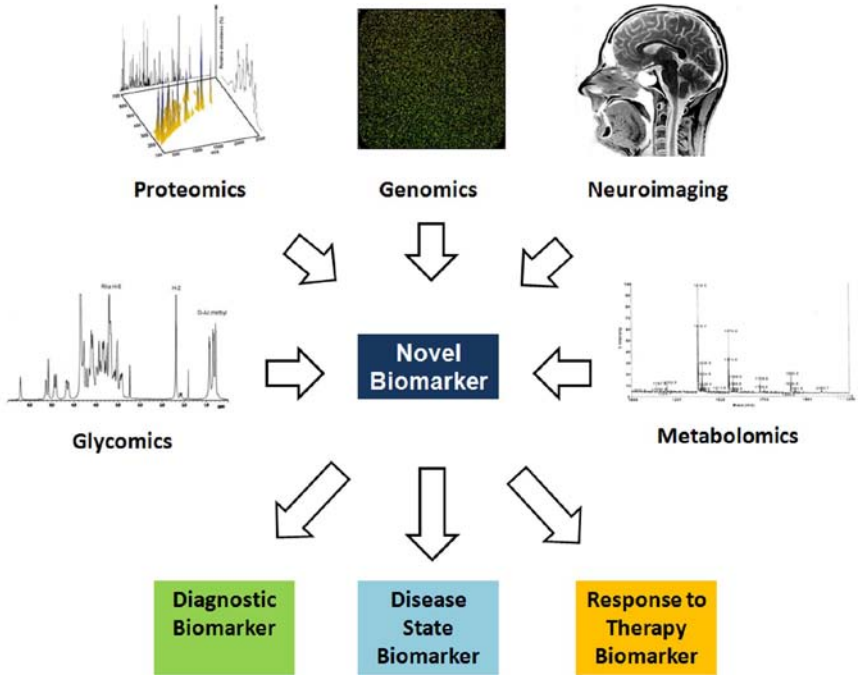

Figure 1. Flow-chart showing multifaceted approach towards biomarker development.

to assure reproducibility. In the end, the most definitive confirmation of any genomic or proetomic biomarker will be in correlating the improvement of biomarker endpoint with clinical improvement in longitudinal studies. Biomarker identification in neurological disorders has been hindered by the unique cellular and phenotypic complexity of the brain. Although the results of blood and CSF biomarker studies for neurodegenerative diseases show promise, it is possible that a combination of biomarkers developed using the modern high-throughput techniques, including genomics, proteomics, metabolomics and glycomics, may be needed, in concert with neuroimaging approaches, in order to develop a viable biomarker (Figure 1). Such systems biology approaches will lead to identification of new biomarkers at multiple levels, namely DNA, RNA, protein and small molecules.

In the development of novel biomarkers a combination of several approaches, such as genomics, proteomics, metabolomics, glycomics and neuroimaging may be needed for a useful clinical application. In the end, development of several biomarkers might be needed to fulfill various applications.

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