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Word count: 2970

Conflict of interest statement: There is no conflict of interest.

Abstract

Multiple sclerosis (MS) is chronic, inflammatory disease of the central nervous system that mainly affects young adults and is characterized with dissemination of demyelinating lesions in time and space. It is well known that MS is very heterogeneous disease, so biomarkers that would reliably determine disease course, outcome or treatment response in early stages of the disease (preferentially clinically isolated syndrome) are desperately needed. Genome-wide expression analysis represents the profile or imprint of all genes in a certain tissue or cell population in certain time point. Therefore, as human genome is entirely known, it is possible to analyze any given human gene in any given context. This review will discuss results and possible applications of gnome-wide expression studies in brain tissue and blood samples of MS patients.

Key words: Multiple sclerosis, biomarkers, genomics, microarrays

Introduction

Multiple sclerosis (MS) is chronic, inflammatory disease of the central nervous system (CNS) that mainly affects young adults and is characterized with demyelination. Although MS is pathologically and clinically heterogeneous disease, in 85% of patients it starts with acute or subacute episode of neurological dysfunction attributable to one or more foci of demyelination, which is called clinically isolated syndrome (CIS). In 21% of patients, CIS manifests as optic neuritis, 46% myelopathy, 10% with brainstem symptoms and in 23% with multifocal symptoms.[1] One of the main characteristics of MS is high heterogeneity in final outcome between patients, with benign or even asymptomatic disease on one end and highly aggressive, malignant disease on the other end of the spectrum. Therefore biomarkers that would reliably determine disease course, outcome or treatment response in early stages of the disease (preferentially CIS) are desperately needed.

Biomarkers

Biomarkers are very important indicators of normal or abnormal biological processes. By definition, biological markers or biomarkers are characteristics that can be objectively measured, and serve as indicators of normal biological process, pathological process or pharmacological response to therapeutic intervention.[2] Potential implications for biomarkers are numerable, they can be used in disease diagnosis and monitoring and determining early efficiency of treatment. As well, they are invaluable in early disease detection, staging and prognosis.

MS is heterogeneous disease in rate of progression, clinical symptoms, specificity of immune response and pathology of MS lesions, so it is to be expected that certain biomarker will reflect just one of many pathogenetic mechanisms involved.[3] Therefore patient stratification will be possible only with defined group of biomarkers so relative contribution of each of different pathogenetic mechanisms can be determined.[4] It is also important that every biomarker is validated in different, independent cohorts in prospective multicentric studies.

Validation criteria for each biomarker are defined according to the purpose for which the biomarker in question is created and should be standardized in different cohorts with following goals:[4]

1) Clinical relevance: biomarker has ability to follow changes in the pathological process and/or therapeutic intervention in relatively short period of time
2) Sensitivity: ability that the biomarker can be measured with precisely enough and that change of biomarker reflects the change of clinical endpoint
3) Specificity: ability of biomarker to identify persons with certain disease or response to certain therapeutic intervention
4) Probability of falsely positive and falsely negative cases: defined as situation in which change of the biomarker does not reflect change of the certain clinical endpoint
5) Accuracy, precision, reproducibility and variability of the laboratory test which measures the biomarker

Currently there is no MS biomarker that fulfills all of the above mentioned criteria.[5]

Genomics

The development of genomics has for the first time made possible to overcome many of above mentioned problems in development of MS biomarkers. With this method it is possible to determine the profile or imprint of all genes in certain tissue or cell population in certain time point. As human genome is entirely known, it is possible to analyze any given human gene in any given context, so it is possible to understand complex molecular interactions, discover biomarkers that correlate well with clinical signs and discover new therapeutic possibilities.[6] Genomic analysis is performed with microarrays that contain known oligonucleotides of very high density, attached to surface in a specific order of very high density. DNA microarrays can be divided in two groups according to DNA type: oligo microarrays contain synthesized oligonucleotids, while cDNA microarrays contain complementary DNA molecule cloned or amplified with PCR.[7,8] The main objection of this method is that it is not hypothesis driven. However, because genetically MS is very complex disease and there is great heterogeneity in clinical picture, genome-wide expression studies have clear advantage over conventional studies, with possibility of forming new hypotheses.[6] During the interpretation of data obtained with these studies, researcher should always bear in mind intra- and interindividual variations between patients[9]. Furthermore, there always exists a question of technical variability, namely use of different microarrays and manufacturer specific protocols, which may influence the final results of expression profiling experiments. Recent 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.[10] Alternatively, using two or more microarray platforms in analysis of the same samples and selecting the most reproducibly differentially expressed genes as biomarkers, could provide a

way to reduce the influence of inter-platform technical variability on the process of biomarker selection. 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.

Recently guidelines have been set out for improvement of reliability of microarray results:[6]

1. Microarray results should be confirmed with alternative methods (real time PCR). However shortage of confirmatory tests does not reduce the importance of microarray results if the microarray experiment is of sufficient quality.
2. Microarray results should always be replicated in an independent cohort of patients. As an alternative one may use biostatistical methods.
3. All samples should be collected at the same time of the day, and time between sample collection and processing should be standardized.
4. If one is doing blood samples analysis, distribution of main mononuclear cell lineages should be analyzed.
5. Relapses, infections and drugs should not interfere with the results.
6. All samples should be collected by standardized method.
7. Analysis should include and dose genes which are near the cut-ff values.
8. Microarrays provide us with great amount of data, so every researcher should make a repository of all available data so the rest of scientific community can gain access to the results. Repositories like this should be in concordance with MIAME (Minimum Information About a Microarray Experiment) guidelines.[11]

What have we learned from genome-wide expression studies in brain tissue of MS patients?

The first study using gene microarrays on brain tissue of MS patients was published in 1999.[12] This study investigated expression profiles differences of more than 5000 genes between normal brain tissue and acute MS plaques and found expression differences in 62 genes, among them Duffy chemokine receptor, interferon regulatory factor-2 and TNF alpha receptor2. Following this study, many investigators used gene microarrays on brain tissue of MS patients with aim of discovery of new biomarkers[13,14], new pathophysiological mechanisms or new therapeutic targets.[9,15-22]

Most of these studies are performed on postmortem tissue, because biopsies are very rarely performed in MS patients. As degradation of RNA happens relatively quickly after death, it is necessary to process brain tissue very quickly in order to obtain RNA of high enough quality for DNA microarrays. Despite this RNA can be isolated from postmortem samples up to 20 hours after death, although short time does not guaranty RNA of high quality.[23] It should be emphasized that postmortem tissue, despite all disadvantages, is a very important source for gene microarrays, especially when investigating new pathogenetic mechanisms and discovery of new therapeutic targets.[23] It is always necessary to carefully characterize the tissue from which RNA will be isolated, immunohistochemistry with markers for demyelination, infiltration and gliosis are all required for good interpretation of results. All these tasks are almost impossible to fulfill, but they should be taken into account when analyzing the data.

In order to identify genes which are crucial in MS pathogenesis, Whitney and coworkers have compared gene expression profiles in MS lesions and brains of EAE mice with normal white matter.[12] Altogether 2798 genes were compared and one of the most important findings was that 5-lipooxigenase, a key enzyme in the biosynthesis of proinlammatory leukotrienes, is overexpressed in MS lesions and brains of EAE mice. Although this finding is not specific to

MS, it emphasizes the importance of proinflammatory activity in demyelinating process and suggests the possible role of antiiflammatory therapy in MS.

Tajouri and coworkers have compared RNA expression profiles of chronic active and acute MS lesions between themselves and in comparison with normal white matter.[15] These authors have identified 139 differentially expressed genes between MS lesion and normal tissue, 69 of them showed same direction of expression in both chronic active and acute MS lesions, while 70 genes showed different direction of expression between chronic active and acute MS lesions. This study revealed genes with already known role in MS pathogenesis like myelin basic protein, glutathione-S-transferase M1 and different growth factors. As both, chronic and acute lesion, had similar expression profiles, it has been suggested that quantitative rather than qualitative differences in gene expression, define progression from acute to chronic active MS lesion.

Further studies have confirmed differences in gene expression between chronic active and inactive MS lesions, in their edges as well as in the central parts.[16] The most differentially expressed genes were genes implicated in inflammatory response, apoptosis related and stress-induced genes. Major differences in gene expression occurred between the lesion margin and lesion centre in active lesions (57 and 69 genes differentially expressed, respectively), whereas the margins and centres of silent lesions showed markedly reduced heterogeneity (only 11 and two genes differentially expressed, respectively).[17]

Microarray analysis of MS lesions obtained at autopsy revealed increased transcripts of genes encoding inflammatory cytokines, particularly interleukin-6 and -17, interferon-gamma and associated downstream pathways. Comparison of acute lesions with inflammation versus 'silent' lesions without inflammation also revealed differentially transcribed genes.[18] Some of these

differentially expressed genes were chosen as therapeutic targets in EAE model of MS, and these results have confirmed the importance of microarray analysis research.

Microarrays on autopsy samples of patients with secondary progressive MS provided molecular evidence of a continuum of dysfunctional homeostasis and inflammatory changes between lesions and normal appearing white matter (NAWM), and supported the concept of MS pathogenesis being a generalised process that involves the entire CNS.[19] When comparing gene expression in NAWM of postmortem brains of MS patients, there is increased expression of genes involved in maintenance of cellular homeostasis, and in neural protective mechanisms known to be induced upon ischemic preconditioning.[20] When comparing expression levels of 33,000 characterized genes in postmortem motor cortex from MS brains, compared with controls, 488 transcripts were found to be decreased and 67 increased. Twenty-six nuclear-encoded mitochondrial genes and the functional activities of mitochondrial respiratory chain complexes I and III were decreased in the MS motor cortex, which was specific for neurons. In addition, pre-synaptic and postsynaptic components of GABAergic neurotransmission and the density of inhibitory interneuron processes also were decreased in the MS cortex.[21] These data supports a mechanism whereby reduced ATP production in demyelinated segments of upper motor neuron axons contributes to progressive neurological disability in MS patients.

One of the advantages of microarray technology is that specific group of genes related to a specific pathway or process can be analyzed separately. In this context, a focused endothelial cell biology microarray, capable of detecting changes in expression of 113 blood/brain barrier-specific genes showed 52 differentially expressed genes in MS lesions compared to NAWM and healthy controls.[22]

What have we learned from gene expression studies on human blood samples in MS?

One of the most important factor that one should take into account when interpreting results of gene expression studies on human blood samples is great interindividual and time variation in gene expression in healthy subjects.[24,25] These variations are dependent on cell composition of the blood, sex, age and time of the day when samples are taken. The partly intrinsic variations can be caused by genotype differences, epigenetic phenomena or environmental and nutritional factors. That is why as many as possible variables should be controlled during processing of samples for microrarray studies.

On the other side, gene expression studies on human blood samples have many advantages. Blood is a very accessible sample to take so it is possible to perform analysis on larger number of patients than in studies using brain tissue. Results obtained with this method can give insight into drug efficacy much earlier, because blood is one of the first tissue with which the drug comes into touch with. However it should be kept in mind that blood also has many disadvantages, mainly because it reflects influence of other factors not pertinent to the disease process itself.[26]

There are two possible ways how to isolate RNA from blood samples. One is isolation of peripheral blood mononuclear cells (PBMC) from the whole blood by Ficoll gradient, and the other one is isolation of RNA from the whole blood with Paxgene tubes. Both procedures have their advantages and disadvantages.

Gene expression studies on human blood samples in MS have been used for diagnostic (differentiation of MS patients from healthy controls, differentiation different MS types, differentiation of treated MS patients from untreated MS patients), prognostic (differentiation of MS patients in relapse from patients in remission) and therapeutic (differentiation of treatment

responders form treatment failures) purposes. One of the first studies aimed at discovering specific expression patterns in blood of patients demonstrated that cDNA microarray technology could be used to identify large-scale abnormal gene expression patterns in the peripheral blood of MS patients. This study identified 34 differentially expressed genes in patients with a relapse-remitting form of the disease when compared to healthy individuals. Significant increases were observed in molecules involved in T-cell and B-cell activation (LCK, CAMP responsive element modulator, IL-7 receptor), and degradation of extracellular matrix (MMP-19 or RASI-1), and significant decreases were observed in proteins that serve as chemokine receptors (STRL 22), are involved in apoptosis (DNA fragmentation factor-45) or in humoral immune responses (immunoglobulin heavy chain Gm marker).[27] Further to this study, Bomprezzi and collaborators identified a set of 53 genes differentially expressed in MS patients that can be used to predict the disease state in an independent test set.[13] As well the findings of this study supported the significance of autoreactive T cell activation as a primary pathophysiological event in MS. In an effort to develop a biomarker capable on not only successfully diagnosing patients with MS, but also differentiating between those experiencing a relapse from patients in remission, Achiron and coworkers identified 721 genes involved in activation of T-cells, epitope spreading and evasion of immune regulation in patients with acute MS relapse, when compared to MS patients in remission.[28] As well, this study broadened the number of differentiating genes between MS and healthy controles to 1,109 (589 overexpressed and 520 underexpressed) mainly involved in T-cell expansion and activation, inflammatory stimuli (cytokines and integrins), epitope spreading, and survival advantage leading to aberrant apoptosis.

Much larger number of studies tried to investigate changes in peripheral blood expression in response to immunomodulatory therapy. A study performed in patients receiving interferon beta

therapy discovered that its action is not purely anti-inflammatory[29], and further studies identified 21 genes differentially expresses in blood of MS patients treated with interferon beta after 3 and 6 months of therapy. Out of the 21 differentially expressed genes, 9 possessed interferon responsive promoters. No significant change in expression of Th1 or Th2 related genes was observed.[30] These studies showed the complexity of expression changes in response to interferon therapy, and indicated the effect it exerts on cell migration, matrix degradation, proliferation, cell cycle control, differentiation, cell processing and presentation, apoptosis and cytokine and chemokine regulation. Trying to identify genes which could predict a favorable response to interferon therapy, a group of authors showed that interleukin 8 might be useful in predicting which patients will show a positive response.[31] These studies also showed that interferon beta induces expression of genes in a selective and time dependent manner, indicating its possible role in monitoring response to therapy.[32] This is especially true for genes involved in antiviral response, which are induced 1-4 hours following interferon beta administration. Similar expression pattern can be observed in genes involved in interferon beta signaling and lymphocyte activation.

Meta-analysis of expression profiling studies conducted using blood samples of patients with MS indicated 15 potential biomarkers which are expressed during the entire course of beta interferon treatment and which can serve as biomarkers of response to therapy. These include EIF2AK2, IFI6, IFI44, IFI44L, IFIH1, IFIT1, IFIT2, IFIT3, ISG15, MX1, OASL, RSAD2, SN, XAF1 and transcript represented by the Affymetrix probe 238704_at.[33] These biomarkers were all formerly identified as being indicative for IFNB activity.

More recently, it has been shown that after second day, one month, 12 months and 24 months of initiation of interferon therapy there are 42, 175, 103 and 108 differentially expressed genes,

respectively. *MS4A1* (CD20), a known target of B-cell depletion therapy, was significantly downregulated after one month and *CMPK2*, *FCER1A*, and *FFAR2* appeared as hitherto unrecognized multiple sclerosis treatment-related differentially expressed genes that were consistently modulated over time.[34]

Another drug approved for RRMS treatment is glatiramer actetate (GA). Gene expression studies on human blood samples have shown that GA alters expression of 480 genes within 3 months of treatment; 262 genes were up-regulated, and 218 genes were down-regulated. The main convergent mechanisms of GA effects were related to antigen-activated apoptosis, inflammation, adhesion, and MHC class-I antigen presentation.[35]

Conclusion

So far, microarrays performed on brain tissue of MS patient provided us with important findings related to MS pathogenesis. Identification of endogenous protective mechanisms in MS brains can lead to development of new drugs which could slow the progression of disability.[23]

The expression signature of MS as detected in PBMC can be used in assessment and monitoring MS patients, discovery of new pathogenetic mechanisms and monitoring of response to treatment. However one has to bare in mind, that in a complex disease like MS both multiple interactions of different components of the immune system *in vivo*, and the complexity of the intracellular pathways must be considered in the interpretation of microarray experiments.[36]

In both cases, future studies with larger number of well defined patients will give us a better insight in molecular basis of heterogeneity of MS.

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