

REVIEW

Pros and cons of microarray technology in allergy research

M. Benson*, M. Olsson*, M. Rudemo†, G. Wennergren* and L. O. Cardell‡

*Pediatric Allergy Research Group, Queen Silvia Children's Hospital, Gothenburg, Sweden, †Department of Mathematical Statistics, Chalmers University of Technology, Gothenburg, Sweden and ‡Laboratory for Clinical and Experimental Allergy Research, Department of Oto-Rhino-Laryngology, Malmö University Hospital, Malmö, Sweden

Introduction

It is not known how many of the 30 000 human genes are involved in allergic disease. DNA microarray technology allows analysis of expression of all these genes in tissues or cells. This could lead to disease models that simultaneously describe all changes in gene expression in allergic disease. This may, in turn, result in identification of new disease mechanisms, diagnostic markers and therapeutic candidates. On the other hand, interpretation of the results can be confounded by the large number of analysed genes and by the methodological complexity. This review aims to give an overview of DNA microarray technology and applications in allergy research. Since there are few microarray studies in allergy, examples from other fields will also be given. Moreover, the analytical process, problems and future directions will be discussed. We have aimed at avoiding technical details and instead suggest references for more elaborate descriptions.

DNA microarray technology

There are many different microarray systems and they have been described in detail elsewhere [1]. However, the basic principle is the same for most microarrays. Unknown mRNA from the sample to be analysed is hybridized to an ordered array of DNA sequences corresponding to known genes or expressed sequence tags. Every mRNA, or transcript, is labelled with a marker, e.g. a fluorescent dye. The location and intensity of the resulting signals give an estimate of the quantity of each transcript in the sample. A DNA microarray can contain DNA sequences corresponding to more than 30 000 human genes, i.e. the genome, ordered on a miniature glass slide (Figs 1 and 2). It is thus possible to measure all transcripts in the sample, i.e. the transcriptome. Microarrays can also be used to search for DNA polymorphisms (single nucleotide polymorphisms, or SNPs), but this review will focus on measuring mRNA, i.e. gene expression profiling.

Examples of microarray studies in allergy research and other fields

DNA microarrays can be used to compare gene expression in cells or tissues taken from patients and controls, or from

patients before and after some form of treatment. The objective has been to identify new disease mechanisms, biomarkers and therapeutic targets. A medline search based on the word 'microarray' in February 2004 retrieved 5296 citations. Many of these were related to cancer research (1588). By comparison, there were few microarray studies in allergy (23). However, several reports will appear shortly in allergy-immunology journals.

Identification of new genes and disease mechanisms

Several different analytical strategies have been tried to identify new genes and disease mechanisms. Early microarray studies were often based on comparisons between a few pooled samples, e.g. from patients and controls. Genes were identified based on criteria such as a twofold difference in expression levels. Since thousands of genes were analysed, this involved a considerable risk of spurious findings. The relevance of identified genes was validated by functional experiments or by examining larger materials with other methods. For example, in a microarray study of pooled nasal biopsies from patients with allergic rhinitis a group of transcripts were described. The corresponding proteins were then analysed in nasal fluids from the same patients. Since this could be done in individual samples, the results could be tested statistically [2, 3]. In recent studies of atopic dermatitis and nasal polyps, individual, rather than pooled, samples have been examined. Selected genes have been verified with real-time PCR or immunohistochemistry [4–6]. In these studies distinctive gene expression patterns were also identified, e.g. a Th2-like pattern in atopic dermatitis. Interestingly, microarray analyses of Th2 cells from patients with allergy also show up-regulation of gene families such as chemokines and chemokine receptors [7, 8]. Microarray analysis has been used to elucidate the effects of specific cytokines relevant to allergy. For example, the Th2 cytokine IL-13 was shown to induce distinct transcriptional programs in human airway epithelial cells, smooth muscle cells and fibroblasts [9]. These studies indicate a unique feature of microarrays, i.e. to identify whole groups of functionally related genes, rather than individual genes. In the same way, whole pathways can be examined; in a study of allergic rhinitis decreased expression of TGF- β induced genes was found [10] (Fig. 3). Recently, a new pathway was identified in experimental asthma, i.e. genes associated with L-arginine metabolism [11].

Another approach to identify relevant transcripts is to combine microarray data with genetic studies. Linkage scans have identified chromosomal regions that may contain

Correspondence: Mikael Benson, Pediatric Allergy Research Group, Queen Silvia Children's Hospital, SE-416 85 Gothenburg, Sweden.
E-mail: mikael.benson@vgregion.se



Fig. 1. A DNA microarray GeneChip. These can be used to analyse up to 47 000 transcripts (image courtesy of Affymetrix).

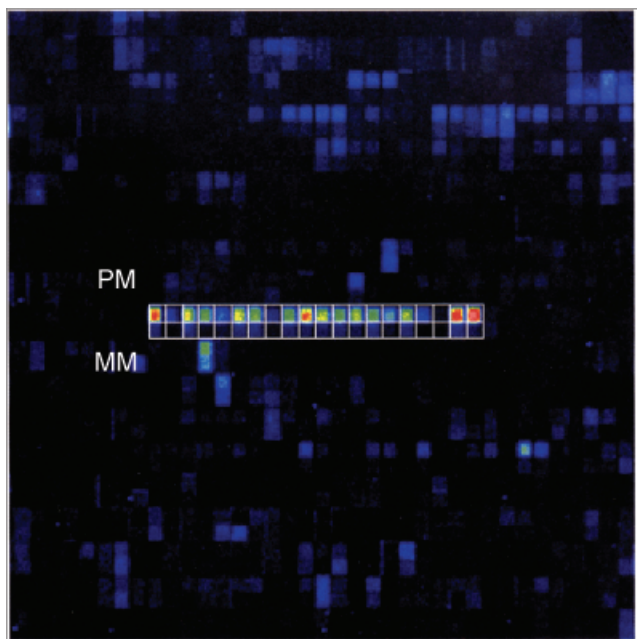


Fig. 2. Magnification of a DNA microarray surface after hybridization with RNA. Each cell contains oligonucleotides that correspond to one gene. These are arranged in pairs consisting of one perfect match (PM) and one control mismatch (MM) oligonucleotide. Each cell contains millions of copies of the same oligonucleotide. The colour intensities of the probe cells are proportional to their hybridization intensities (HuGeneFL Array, Affymetrix).

candidate genes for allergic disease. Since such a region may contain hundreds of other genes, identification of the actual candidate gene is difficult. However, polymorphisms in regulatory elements of candidate genes are common and may lead to altered gene expression [12]. Such alterations may be detected using DNA microarrays. Thus, the search for

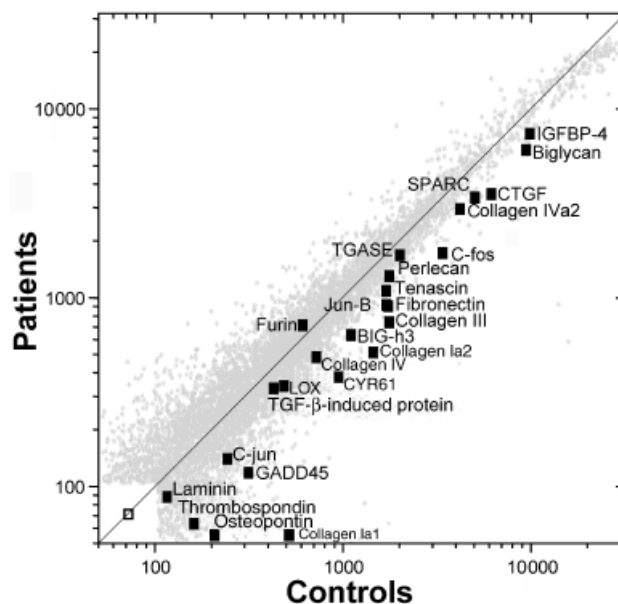


Fig. 3. Scattergram showing expression levels of genes induced by Transforming growth factor (TGF)- β in nasal biopsies from patients with allergic rhinitis and healthy controls [10]. The diagonal line is the line of identity.

candidate genes may be narrowed down by searching for changes in gene expression in chromosomal susceptibility regions with DNA microarrays. In a very elegant study this approach led to identification of complement factor 5 as a candidate gene in a transgenic model of allergen-induced airway hyper-responsiveness [13].

Classification of disease

Gene expression patterns can be used as 'fingerprints' to distinguish between different forms of disease. This has been mostly done in cancer research, i.e. to classify different tumors [14]. However, in a study of asthma, a combination of genes was shown to more accurately discriminate between the asthmatics and healthy controls than IgE [15]. The same principle could be applied to select which drug is most effective in treating different individuals with the same disease.

Studying the effects of drugs

Treatment with drugs, such as glucocorticoids (GC) is effective but may be complicated by side effects. Interestingly, microarray studies of airway cells and tissue show that the beneficial effects of GC may, at least in part, depend on up-regulation of anti-inflammatory genes. It is possible that such genes may provide clues to anti-inflammatory drugs that lack the side effects of GC [6, 16].

Analytical process

A DNA microarray study is a multi-disciplinary effort involving molecular biologists, bioinformaticians, and other experts, such as clinicians and immunologists. The complexity of the microarray data emphasizes the need for well-defined clinical materials and established experimental methods to

verify the results. The analytical process consists of different phases, i.e. planning, obtaining samples, RNA extraction, DNA microarray analysis, bioinformatic analysis and validation of selected genes. There are public databases on the Internet that can be useful for the different phases (Table 1). To give an idea of the practical aspects of a microarray study, these phases will be discussed, and some examples given.

Planning the study

This phase should attempt to prepare for the whole analytical process. Important questions are: Which biological questions should be answered? Which type and size of material is required? How much high-quality RNA can be obtained? Should individual or pooled samples be analysed? Should duplicate microarray experiments be performed? Is extra material needed for validation studies with other methods? How should the samples be handled? How should microarray data be stored and analysed? How should validation studies be performed? There is no consensus on these issues. For example, pooling of samples have often been used in early microarray studies. However, for clinical studies, where variation between patients is typically the crucial source of variation, our experience is that pooling should only be employed if it is impossible to get enough RNA otherwise. Similarly, if there is a choice between use of duplicate chips and use of single chips with twice as many patients we prefer the latter alternative. The microarray experiment can be seen as a tool to screen for new genes that can be further analysed in other settings. This means that experiments sometimes need to be performed sequentially, and it is advantageous to include this aspect from the start of the study. All questions in the planning phase have impact on each other and all collaborators should be involved.

Obtaining samples, RNA extraction and microarray analyses

These issues may appear trivial, but even small errors will have large effects on the results. Standardized protocols for different settings are constantly developed and available from manufacturers and public websites. Pilot studies may be needed to determine if the quantity and quality of RNA is sufficient. Protocols for quality controls vary in different systems. Microarrays from Affymetrix contain internal controls such as spiked levels of different genes. Comparisons between oligonucleotide microarrays and real-time PCR have shown good correlations [17].

Bioinformatic analysis

It should be emphasized that microarray bioinformatics is developing into a separate speciality and that the success of microarray experiments is greatly helped by collaboration with bioinformaticians [18]. The analysis of microarray data can be divided into different steps:

(a) *Storage of data.* The data has to be stored safely and yet accessible for repeated analyses. Standards for this has been described by MIAME set of protocols [19].

(b) *Normalization of data.* The complexity and scale of microarrays may lead to considerable methodological variation. For example, genes with low expression levels typically show a larger coefficient of variation compared to genes with high expression levels. Low-abundance genes, are therefore, more likely to be declared differentially expressed when comparing two samples. To compensate for this, the data have to be normalized. This is an important and complex step, for which several methods have been described [20].

(c) *Identification of relevant genes.* This can focus on either finding individual genes or groups of functionally related genes. In the first case, the simplest approach is to use a pre-defined cut-off, such as a twofold ratio in expression. This disregards the variability of the data, and may result in a large number of spurious identifications. Different statistical methods that take into account the variance of the data give a smaller and more specific selection of differentially expressed genes [21]. The list of differentially expressed genes can then be searched empirically or using public databases that describe the functions of individual genes. Another approach to identify relevant genes is to combine the microarray data with some other form of biological information. For example, the expression levels of the genes in the microarray experiment can be correlated to some biomarker, such as IgE or symptom scores. Another example is to search for differentially expressed genes that are located in chromosomal susceptibility regions. This approach proved successful in a transgenic model of asthma [13]. In our own experience, the same approach applied to human allergic rhinitis did not lead to identification of candidate genes that could be validated in a new material. This may depend on the complexity of human allergic disease. Indeed, identification of genetic variants with linkage studies have proven difficult for complex diseases, as each variant only contributes modestly to disease [22].

Finding groups of functionally related genes may be advantageous since the likelihood of biological relevance increases if many genes with similar function are co-regulated. The search can be based on finding genes that have correlated expression levels. Cluster analysis is a common method to identify co-expressed genes. The most common form of

Table 1. Public databases for microarray experiments

Entrez is a linked system of databases to find information about individual genes and literature references (<http://www.ncbi.nlm.nih.gov/>).

One of the databases, Omnibus, contains freely searchable data from more than 500 microarray experiments (<http://www.ncbi.nlm.nih.gov/geo/>).

BASE (<http://base.thep.lu.se/>) aims to provide all the tools needed for data storage, quality control, normalization and statistical application in a web-based application (Lao).

Minimum information about a microarray experiment – MIAME gives standards for microarray experiments are described at <http://www.mged.org/Workgroups/MIAME/miame.html>.

The Gene Ontology Consortium and Pubgene and provides tools for automated grouping of functionally related genes (<http://www.geneontology.org>, <http://www.pubgene.org/>). GenMAPP may be used to identify activated biological pathways in gene expression data (<http://www.genmapp.org/>).

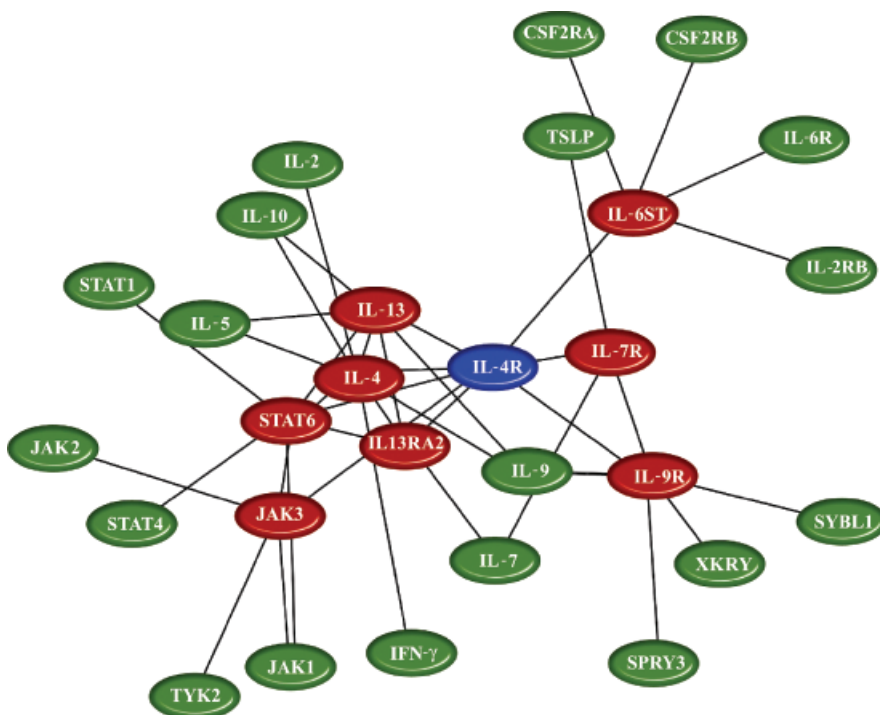


Fig. 4. Literature network of genes related to the IL-4 receptor (IL-4R). The gene symbol IL-4R was submitted to PubGene to identify genes that are co-cited with IL-4R in the literature. Lines connect genes that have co-occurred more than once in the literature. Blue symbol = IL-4R, red symbols = first-degree neighbours to IL-4R in the literature, i.e. co-cited in PubMed articles, green symbols = second degree neighbours, i.e. co-cited with first-degree neighbours. Only frequently mentioned genes are shown in this graph, for clarity (image courtesy of Eivind Hovig).

cluster analysis is hierarchical clustering, in which an iterative algorithm draws up a tree in which the lengths of the branches correspond to the degree of similarity between the genes. Other methods to identify patterns of gene expression, such as principal component analysis and neural networks, have been used to classify tumors [14]. Principal component analysis may be of interest because the relations between different variables can be described in graphic disease models [23]. Another approach to identify functionally related genes is to organize differentially expressed genes in functional groups based on public databases, such as Entrez. Gene Ontology Consortium has assigned functional codes to different genes based on other databases and literature searches. These codes can be used for functional grouping of genes. There are also web sites designed to identify biological pathways, such as GenMAPP [24]. Pubgene has a unique approach to identify functionally related genes. The underlying assumption is that if two genes are cited in the same article this reflects a biological relationship. The relationship can be visualized by drawing a line between the genes. Pubgene has searched through more than 10 million abstracts in Medline to make a network, in which each gene is a node connected by co-citation lines. This network can be used to make graphic models that give a visual overview of functionally related genes (Fig. 4). It is also possible to identify diseases or biological processes associated with such groups of genes. In the context of microarray studies, the co-citation network can be used to find relations between differentially expressed genes [25].

Validation of selected genes

There are two methods for independent confirmation of microarray data: *in silico* analysis and laboratory based analysis [26]. The *in silico* method compares array results with

information available in various databases (Table 1). It is, however, important to verify changes in gene expression with another laboratory method, such as real-time PCR. Since changes in gene expression do not necessarily correspond to protein levels, quantitative analyses, e.g. with ELISA are called for. If the DNA microarray analyses are performed on mixed cell populations, such as tissues, immunohistochemical examination is needed to determine the cellular origin of the regulated gene. Experiments to examine the functional role of the genes may further strengthen the findings.

Cons

Although microarray technology may result in significant scientific advances, there are several concerns. The cost of chips means that the number of repeated observations is usually very low. This, in turn, involves a considerable risk of spuriously identified genes. There is a lack of standards for data collection, analysis and validation. Most microarray systems require a few micrograms of RNA. Obtaining RNA of sufficient quantity and quality is a challenge in many clinical studies.

The size of the datasets and the complexity of the analytical process involve risk of over-interpretation of the results. For example, in one study clustering was used to classify B cell lymphomas [27] but this could not be reproduced [28]. If tissue samples are compared, the gene expression data represents mixtures of cells types, of which only a few may be relevant for the disease. Conversely, *in vitro* studies of selected cell populations may not be representative of *in vivo* findings. Gene expression data may be confounded by cross hybridizations between transcripts with high sequence homology, inaccurate sequence database annotations or the

presence of splice variants. Moreover, mRNA levels may not be proportional to protein levels because of different factors, such as variable efficiency of mRNA translation.

Future directions

Microarray experiments in allergy research have resulted in identification of new transcripts for further studies with more established experimental methods. It is possible that coming studies will help to find new disease mechanisms, biomarkers and perhaps therapeutic targets.

It is noteworthy that the studies performed so far have implicated parts of the transcriptome that encode many different functional systems. These parts have been related to the immune system, but also systems not usually associated with allergy such as electrolyte transport or metabolism. This diversity is consistent with linkage studies that have described more than twenty chromosomal susceptibility regions for allergic disease. Coming microarray studies are likely to lead to identification of more new parts of the transcriptome. This leads to the question if allergy can be understood by detailed characterization of individual parts, independent of each other. Or can the data be integrated into some form of pathogenic model for allergic disease? These questions are not unique to allergy research. The very size of the data generated by microarray experiments in various fields suggests that complex systems may not be understandable by investigation of their respective parts in isolation. This reflects the basic principle that everything in a living cell is interconnected to everything else. It may be possible to address this complexity by treating gene expression data as a network. Biological networks have characteristics that can be described with bioinformatic methods [29]. In a recent article in *Science*, such a method was applied to model the structure of a gene expression network. This model included basic functional parts of the transcriptome, such as signalling and metabolism [30]. It may become possible to make experimental studies of such models, e.g. to understand the effects of an external stimulus. If similar principles are generally applicable it may be possible to make disease models that incorporate all transcriptomal changes in allergy. Such models might be dissected to examine different parts, e.g. the immune system. Perhaps it may also become possible to examine the effects of different drugs or what happens if different components of the transcriptome are changed.

Developments in research fields other than allergy may become relevant for microarray research in allergy. For example, the study that described gene expression data as a network was an *in silico* experiment based on more than 3000 different microarray studies gathered at the Stanford Microarray Database (SMD). SMD aims to provide the research community with searchable data from microarray experiments [31]. The group that constructed PubGene, i.e. the co-citation network to find functionally related genes, validated their method with data from SMD [25]. In a recent study, a research group in Greece used microarray data from SMD to identify new transcription factors in T lymphocytes [32]. It is of note, however, that in February 2004 less than 15% of the microarray data in SMD was publically available. On the other hand, researchers can agree to make a meta-analysis of

microarray data collected in different centres. This approach led to identification of genes that were differentially expressed in four published microarray studies of prostate cancer [33]. To our knowledge, similar *in silico* experiments have not been performed in allergy research, but may become relevant if more microarray studies of allergic disease are performed and the results made available in public repositories. Alternatively, different groups could agree to collaborate as in the meta-analysis of prostate cancer. It is possible that the scale and complexity of microarray studies will favour collaborative efforts. This may, in turn, emphasize the need for well-defined materials as well as state of the art experimental methods.

New methods to profile protein expression, such as surface-enhanced laser desorption and ionization (SELDI) should be mentioned. Such profiles can be used to diagnose cancer in serum samples from patients [34]. However, the technique requires complementary analyses to define the identity of individual proteins. It is possible that combining SELDI and DNA microarray may help to identify transcripts and proteins of pathophysiological and diagnostic relevance. Attempts to make arrays with capture antibodies on glass slides have also been reported and tried on Th1/Th2 cells [35].

Conclusion

DNA microarray technology is still under development. In combination with other methods, microarrays may lead to new understanding of pathogenic mechanisms in allergic disease. This may require large collaborative efforts and lead to identification of new biomarkers and therapeutic targets.

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