DNA Microarrays as a Tool in Toxicogenomics

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Abstract: Toxicogenomics is an emerging technology that defines the use of novel genomic techniques to investigate the adverse effects of xenobiotic on gene expression. Toxicogenomics is based on the fact that most of relevant toxicological effects of a compound affect directly or indirectly the gene expression. The most common methods to profile gene expression at the transcript level are Northern Blotting and the real-time PCR. While commonly used and well accepted, these techniques are now superseded by new technologies allowing the analysis of the expression for multiple genes simultaneously. DNA microarrays are now developed for simultaneous gene analysis but inherent to such multiple assays, their quantitative aspect and their relevance for toxicogenomics have been questioned. We will review here recent studies on their use for toxicogenomics and examine the possible future of such technology in complementation with the other toxicology methods.

Keywords: Low-density microarray, Quantitative assay, Mechanistic Toxicogenomics, Drug metabolism, Predictive Toxicogenomics, Rat liver.m

INTRODUCTION

Toxicogenomics can be described as a marriage of classical toxicology assays and the emerging discipline of expression profiling, a union between the treatment of laboratory animals and the attempt to extract meaning from hundreds of spot on a DNA microarray [1].

Classical toxicity testing is a major bottleneck in the drug development process [2]. This situation was exacerbated with the advent of combinatorial libraries, high throughput screening robots, and the exponential increase in potential drug target triggered by genomics testing at the stages of early drug development. Because later stages of development can cope with only a limited number of potential compounds, increased input does not result in a higher number of successful candidates, thus it is crucial to identify the most promising ones early on [3]. With increasing costs of new drug development, there is a crucial need to conduct toxicity evaluation as early as possible and on as many potential chemical leads as feasible [4].

Although many companies have implemented high throughput screening procedures for selecting new chemical entities (NCEs) on the basis of their pharmacological efficacy [5, 6] and ADME characteristics (absorption, distribution, metabolism and excretion properties of a compound), tools for the rapid analysis or prediction of side effects of NCEs are still lagging behind [3, 7, 8] During the drug developmental process, undesired toxicity accounts for about one third of compound failures [9]. Therefore, it is clear that new powerful technologies are needed as an alternative to classical toxicological tests for a rapid screening.

While it may take weeks, months or even years before some traditional toxicological endpoints occur, specific changes in mRNA levels is one of the early even which occur within a few hours or days after exposure to chemical compounds. Toxicogenomics is based on the fact that most of relevant toxicological outcomes originate from early changes in gene expression [3, 10]. The concept of applying expression profiling to the field of toxicology has several important implications. First of all, the amount of time and resources required for the toxicological analysis of a compound can be reduced. As a particularly striking example, a standard rodent cancer bioassay includes the analysis of over 1000 animals, cost millions of dollars, and taken almost four years [11]. However, tumorigenic compounds may exert their effects much earlier upon entering their target cells. If their early effects are preceded or accompanied by characteristic and reproducible alterations in gene expression profiles over this much shorter period, it is theoretically possible that characteristic changes in RNA transcript levels could be observed within a few hours, days or weeks of exposure to a tumorigenic compound.

The emerging field of "toxicogenomics" is be defined in this review as the study of toxicological processes at the transcriptome level of a target organ or cell. It seems that DNA microarrays could be very helpful not only to predict drug induced toxicity but also to better understand mechanisms of drugs actions [10, 12, 13]. So, gene expression microarrays are not only useful as screening tool but also for the choice of lead compounds. Another important benefit arises from the fact that toxicogenomics analyses are performed at the molecular level. Assessing the transcript levels of several hundreds to thousands of genes allow to determine how compounds interfere with or affect the molecular machinery of their target cells.

Therefore toxicogenomics data also offer additional insights into cellular mechanisms of toxicity beyond those derived from classical toxicological analyses based on whole organ pathology or survival endpoints. Molecular profiling of toxicants may enhance our basic understanding of some of the major issues in risk assessment.

In order to learn the language of the transcriptome, researchers are currently trying to unravel the relevant

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correlations between changes in transcription profiles of target organ and the respective toxicological endpoints. Such studies have been performed on several defined models (e.g. rat liver, hepatocytes) which have been challenged with a number of reference compounds eliciting well-defined toxicological endpoints [14-18]. The number of published results is limited, as the implementation of toxicogenomics database is mostly found within industrial setting, where such work is subject to intellectual property constraints.

TRANSCRIPTION PROFILING METHODS USED IN TOXICOGENOMICS

Established methods of studying gene expression, such as Northern blotting, RNase protection assays, S1 nuclease analysis and quantitative PCR are inherently serial, involving measuring a single gene at a time and are difficult to automate. Recently emerging techniques, such as differential display [19], serial analysis of gene expression (SAGE) [20] and cDNA- and oligonucleotide-based microarray hybridization were developed to allow gene expression analysis of multiple genes simultaneously [21, 22].

Transcription profiling technologies has been classified as closed and open system [3, 8, 10]. Among the open systems, we found differential display technologies [19, 23], the tag-sequencing methods massively parallel signature sequencing (MPSS) [24], the subtractive hybridization [25] and the serial analysis of gene expression (SAGE) [26]. All these technologies are designed to avoid the limitations of a pre-selected gene set and permit, in theory, the analysis of the whole transcription without any a priori.

The most important closed system used today are DNA microarrays that permits the analysis of transcript levels for the set of genes it displays on its surface. DNA microarrays consist of multiple DNA probes spotted and attached covalently to a modified glass slide [21, 22, 27, 28]. These slides are then hybridized with labeled cDNA prepared from mRNA of different samples and differences in gene expression are quantified (Fig. (**1**)).

DNA microarrays consist of either thousands of genes (high density microarrays) or only a limited number of carefully selected genes (low density microarrays). The use of high density microarrays has the main advantage that the expression level of a large number of genes can be studied simultaneously. However, the major drawbacks are related to the quantification control of such large number of genes, the high cost and the time required for analysis and interpretation of the data.

Low density microarrays, even though they consist of fewer numbers of genes can however allow rapid analysis of gene expression changes associated with chemical exposure, and are a fast, reliable and a cost effective method. There are already reports in the literature that describe the use of high density microarrays in drug toxicity [8, 14, 16], but, few studies have been carried out using low density microarrays [12, 29, 30]. Both high and low density microarrays have their advantages and limitations and the choice between them is driven by the aim for which they are used.

Microarrays used in toxicogenomics studies vary widely in the composition and in the number of genes they display, ranging from 43 to 44 000 genes [8]. While more does not necessarily imply better, the relevance of the data for a particular study depends on the composition of the selected gene set. There has been a trend toward using toxicology-focused arrays that display genes selected for their known or suspected involvement in cellular responses to toxic insult [12, 29]. However, in the current situation of this novel discipline, our knowledge about toxicologically relevant gene regulation is still limited. Thus it is an interesting strategy to combine open and closed methods in order to discover novel toxicologically relevant genes and to use them later on well-defined microarrays.

Because they are not restricted to any pre-selected gene set, open transcription profiling technologies can generate very valuable data with respect to mechanistic studies on toxicity. However, because all of these technologies are substantially more laborious than the DNA microarray system, open methods are not well-suited for large toxicogenomics reference database [31-33] and their application has been restricted to only of few compounds.

MECHANISTIC AND PREDICTIVE TOXICOGENOMICS

Gene expression profiling can be employed for two different purposes: mechanistic and predictive toxicogenomics [34]. Mechanistic toxicogenomics uses microarray analyses to visualize gene expression changes related to toxicological endpoints. Identification of gene expression modifications related to known toxicological endpoints allows to investigate and to increase the understanding of mechanism involved in the toxicity.

Microarray analysis has been used to study the gene expression changes caused as a result of toxicity induced by carbon tetrachloride. Holden *et al.* identified 47 genes whose expression was significantly affected by carbon tetrachloride in HepG2 cells. Although a number of these genes have been linked to carbon tetrachloride induced hepatotoxicity, the finding that interleukin-8 (IL-8) was involved was novel [35]. A similar study was reported by Harries et al., who analysed gene expression changes induced by carbon tetrachloride and ethanol. As expected, the two compounds yielded very different expression profiles, reflective of different mechanisms of toxicity [36].

Two reports of Waring *et al.* have generated data for the same set of 15 hepatotoxins, which were analysed for their effects on the transcription levels of around 1000 genes in rat hepatocytes [17] and rat livers [18] providing two significant datasets for comparison of the two model systems. Unfortunately, the comparability of these two datasets is affected by the choice of two different dose treatments. A fixed dose was used for all 15 compounds with hepatocytes; the animals were dosed at a level capable of causing significant hepatotoxicity. The hierarchical cluster analysis revealed few concordances. Given the different study designs, it cannot be agreed as to whether intrinsic differences of the two model systems or the differences in dosing are the main cause for this poor overlap. Microarray

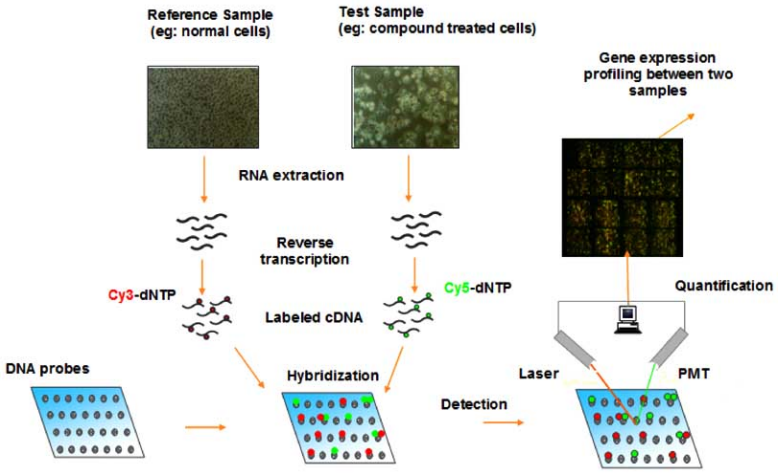

Fig. (1). Overview of microarray procedure applied to gene expression analysis. Reference RNA and test RNA are labelled by reverse transcription with two distinct fluorescent dyes and hybridized to microarray containing robotically printed DNA probes. The slides are scanned with a confocal laser scanner and colour images are generated for each hybridizations with RNA from the test and reference samples. Genes up-regulated in the test sample appear red, whereas those with decreased expression appear green. Genes with similar levels of expression in the two samples appear yellow.

analysis has also been used to identify novel genes associated with toxicity induced by peroxisome proliferating agents [37], arsenic [38], benzo(a)pyrene [39]. In all these studies, the authors identify numerous gene expression changes, which give better understanding of the toxicity mechanism of these compounds.

We have developed a fast and reliable DNA microarray with 59 genes (including 8 housekeeping genes) (Dualchip Rat Hepato, Eppendorf, Hamborg, Germany) from a range of toxic makers and drug metabolism genes that have the potential to be used to follow changes in gene expression levels due to xenobiotic treatment [29]. We used this low density microarray to analyze the gene expression profiles generated in primary cultures of rat hepatocytes exposed to 11 different known hepatotoxicants [12]. These latter were pooled into 4 groups according to their hepatocellular effects: necrosis [isoniazid (ISN) and acetaminophen (APAP)], cholestasis [erythromycin estolate (ERY) and α-naphtylylisothiocyanate (ANIT)], steatosis [tetracycline, 4-pentenoic acid and amiodarone (AM)], and induction of cytochromes P450 (CYP P450) subfamilies [clofibrate (CLO), β-naphtoflavone (BNF), phenobarbital (PB), and zileuton]. The *in vitro* gene expression data generated on microarrays were in good agreement with the reported literature. All the tested drugs generated a specific gene expression profile. Fig. (**2**) shows the hierarchical clustering analysis of the gene expression patterns induced by the 11 hepatotoxicants. A cluster was formed for tetracycline, pentenoic acid and AM. The drugs APAP, ISN, ANIT and ERY were pooled in the same cluster. BNF, PB, zileuton and CLO formed four individual clusters. The clustering analysis revealed that the compounds known to cause steatosis were linked, suggesting that they functionally regulate similar genes and possibly act through the same mechanisms of action. On the other hand, the drugs inducing necrosis and cholestasis were pooled in the same cluster. The drugs arbitrarily classified as the CYP450 inducers formed individual clusters. Despite the low number of genes studied, the gene expression patterns allowed a classification of compounds with similar hepatocellular injuries [12].

While microarrays are useful in mechanistic toxicology, the most exciting application of this technology is the promise to predict potential safety liabilities associated with compounds before any outward manifestation of the toxicity occurred. Such application requires the analysis of a large number of compounds in order to be able to assess the toxic potential of eventually, any unknown compound of interest. It is refereed as "predictive toxicogenomics" in which analyses generate initial reference datasets suited for the categorization of future unknown compounds according to their functional class [1]. Ultimately, it may be feasible to identify a smaller number of genes that may serve as predictive markers for early screening test.

Bulera *et al.* analysed the expression profiles from six hepatotoxins in rat livers [15]. A custom microarray including 1600 rat genes was used in the study, and RNA samples were collected from animals treated at multiple time-points and concentrations. Based on the data obtained on microarray, an unsupervised cluster analysis was used to group the compounds based on the similarity of their expression profiles. In many cases, the expression profiles from the same compounds clustered together regardless of treatment duration. This indicates that individual compounds give unique expression profiles and that this signature can be detected across multiple time-points. In addition, it was possible to identify a blinded sample by clustering its expression profile against expression profiles from other toxins.

The 15 hepatotoxins gene expression database generated by Waring *et al.* [17], was applied to identify the toxic mechanism for a previously unexplored drug lead, an experimental thienopyridine inhibitor of NFκB-regulated adhesion proteins [18]. The gene expression profile for treated rats was compared to their hepatotoxins database. The

clustering analysis revealed that the expression profile for the thienopyridine compound is clustered with two other compounds (3-methylcholanthrene and Aroclor 1254). This paper demonstrate how a database of gene expression profiles could be used to identify a toxicity mechanism associated to new compounds [1].

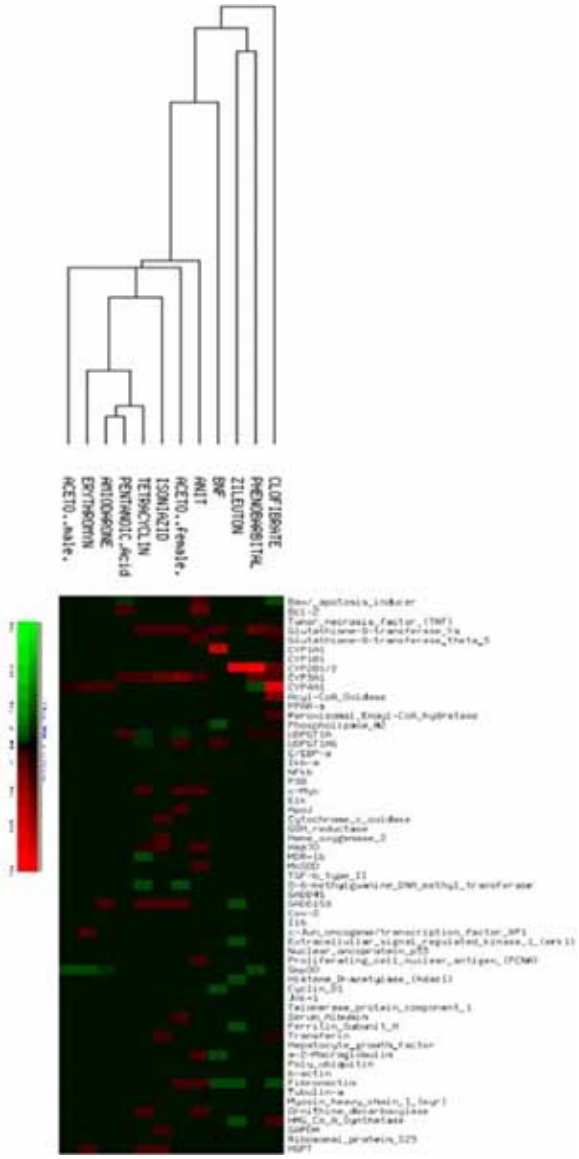

Fig. (2). Hierarchical clustering analysis depicting relative gene expression patterns induced by the 11 hepatotoxicants. A cluster was formed for tetracycline, pentenoic acid and AM. The drugs APAP, ISN, ANIT and ERY were pooled in the same cluster. BNF, PB, zileuton and CLO formed four individual clusters.

Recently, Burczynski *et al.* used HepG2 cells to identify expression profiles from more than 100 compounds. The authors found that when clustering and comparing all of the 250 genes on the microarray, they were unable to distinguish the compounds. However, subsequent analysis identified a small subset of marker genes that provide more predictive accuracy than the larger set [40]. A similar study was performed by Thomas *et al.* In this study, 24 model compounds were clustered based on the overall gene expression patterns from the 1200 genes present on the microarray. As with the study of Burczynski et al., the authors found that a smaller subset of genes provides a more reliable prediction than a larger set of genes. When the compounds were clustered using all 1200 genes, the compounds clustered with a classification accuracy between 50 and 70%. However, the authors identified 12 transcript that gave an estimated 100% predictive accuracy for separating the compounds into their toxicological classes [41]. Both of these studies show the potential for identifying a small subset of marker genes for predicting toxicity. It should be noted that larger microarray studies were needed initially in order to identify the marker genes [1]

MODEL SYSTEMS

While the ultimate goal of toxicogenomics is to generate safe drugs for humans, the majority of studies are performed on rodents despite the fact that the human predictability of standard rodent tests shows only 45% concordance [9]. However, primary hepatocytes are well suited for toxicogenomic studies because they display a certain level of metabolic activity and the liver is a major stage for toxic events [17]. Hepatotoxicity is a common reason for withdrawal of compounds from the market [42]. In addition, the use of cell culture models reduces the animal utilization and need for the synthesis of new compounds on a large scale [43]. However, it is also clear that there are a number of limitations using *in vitro* approaches such as the functional differences observed in primary hepatocytes relative to the intact liver, the absence of interactions with biological entities (e.g. organs, blood) under *in vitro* conditions, the difficulty to select doses and time points which are representative of an *in vivo* situation [12].

DREAMS AND CHALLENGES IN TOXICO-GENOMICS

The results from the first published studies showed that compounds induced changes in gene expression profiles correlate well with the assignment of these compounds to their corresponding mode of action. These studies demonstrated that gene expression pattern is capable of generating the information needed to assign a compound to a mode of action class, which may be exploited for prediction purposes. Several data revealed the importance of the study design that may affect the relevance of the data generated for toxicological processes [1]. Even if the toxicogenomics holds great promise, there are still numerous questions to overcome. The most critical is whether gene expression profiles for a given compounds reproduce consistently from study to study and across different laboratories. An additional challenge is that there is not yet any standard method for performing microarray experiment [44]. An other crucial issues lies in expression monitoring of *in vitro* cell systems since the use of cell culture in toxicogenomics would allow for high throughput screening at an early stage of drug development [1, 12].

CONCLUSIONS

In this review, we highlighted several criteria that are important for toxicogenomics. These are the possibility of

using samples prepared from a low number of cells, the capacity of reliable and quantitative detection of expression of low abundant transcripts, the ability to implement this assay as a high throughput system, and the reproducibility of data across different laboratories. As these challenges are slowly fulfilled, this opens the way for utilizing microarrays as a new tool for increasing the efficiency of the drug discovery.

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