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Toxicogenomics – Applications in Systems Toxicology

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1 INTRODUCTION

A significant increase in mining, refining and manufacturing, as well as the use of products containing chemicals, has taken place during the last one and a half century. Although chemicals are of immense benefit to mankind, some of them, including many commonly used chemicals, pose a serious threat to human health. Significant quantities of various chemicals are present as contaminants in the air we breathe, the water we drink and the food we eat. In addition, significant quantities of various toxic chemicals are found in the environment as well as in various occupational settings as contaminants. Therefore, every human being is at risk of exposure to toxic chemicals capable of resulting in potential adverse health effects. Virtually every organ/tissue in the body has been identified as a target for toxicity resulting from such exposure to toxic chemicals. In fact, chemical exposure has been identified as a major aetiological factor for several diseases in humans, including cancer in various organs and tissues (Loeb and Harris, 2008; Sorahan, 2009). As human exposure to potentially toxic chemicals is almost unavoidable, it has become increasingly necessary to have the capability to effectively detect human exposure to toxic chemicals and, if possible, to predict or even determine the potential for adverse health effects resulting from such exposures. Equally important is the requirement to determine the potential mechanisms underlying the toxicity of chemicals because this may provide an opportunity to develop strategies to intervene or even prevent toxicity resulting from these exposures. This understanding, if possible, should not be confined to a single tissue or organ in the body, but rather be applicable to toxic effects taking place in all organs and organ systems in the body.

Great progress has been made in the last several decades in determining the potential of chemicals to cause toxicity as well as elucidating the potential mechanisms underlying their toxicity. A variety of approaches may be employed to determine whether a chemical is toxic or not. The ultimate goal of a successful toxicity test has to be its ability to predict most accurately the potential of a chemical to cause toxicity in a human being following exposure to the chemical in question. The desired features of an ideal toxicity test are: (i) sensitivity – the test should be highly sensitive so as to detect subtle or pre-clinical toxicity much before the onset of any clinical symptom of toxicity; (ii) non-invasiveness – the test should be applicable in situations of human monitoring for chemical exposure and the resulting toxicity and this is possible at best only if the test can be conducted non-invasively using easily accessible bio-fluids such as peripheral blood, urine, etc.,

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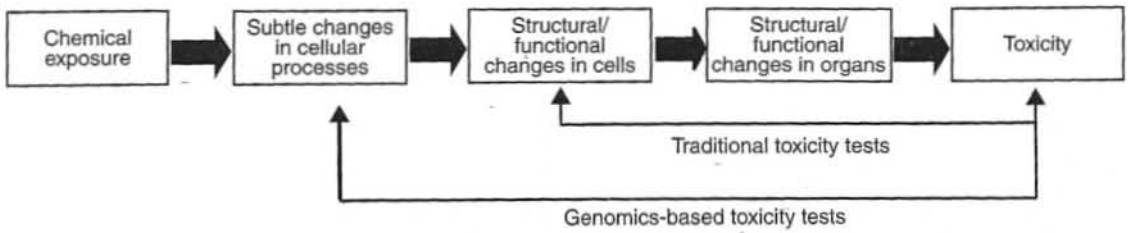

Figure 1. Continuum of events in chemical toxicity. Compared with the traditional toxicity tests, genomics-based toxicity determination can detect toxicity before the onset of clinical symptoms of toxicity.

that may serve as surrogate(s) for target organ(s); (iii) simple – the test should be simple so that it can be performed easily without the need for highly sophisticated equipments or significant technical expertise; (iv) specificity; (v) cost-effectiveness; and (vi) reproducibility. Being able to accurately predict the potential of chemicals to cause toxicity immediately following exposure has the unique advantage of implementing strategies to prevent the onset of toxicity so as to protect the exposed individuals from developing adverse health effect(s).

Various *in vitro* (cell/tissue culture) and *in vivo* (experimental animals) toxicity tests have been developed and are currently being employed to determine the potential of chemicals to cause toxicity (Boulmedarat *et al.*, 2005; Kikkawa *et al.*, 2006). These tests rely heavily on various endpoints pertaining to structural and/or functional damage to cells/organs induced by the toxic chemical. Although the various tests that are currently being employed are capable of detecting toxicity induced by chemicals, they lack one or more of the features described above that would make them ideal to predict and/or determine toxicity. For example, live animals can be exposed to toxic chemicals and histological/biochemical changes taking place in the organ(s) or blood can be determined as indicators of target organ toxicity (Amin *et al.*, 2004; Ohbayashi *et al.*, 2007;Hard, Flake and Sills, 2009). It is to be emphasized that the biochemical and histological changes in the blood and in the target organs, respectively, of the exposed animals are not predictors of toxicity, but rather indicators of injury/damage that has already taken place in response to exposure to the chemicals. Such changes are noticeable only when significant damage, including structural and/or functional damage, to the target organ(s) or tissue(s) has already taken place. Therefore, many of the traditional toxicity testing procedures lack the high sensitivity required to detect early, subtle toxicity (toxicity not associated with clinical symptoms) following exposure to very low concentrations of the toxic chemical (Figure 1). Lack of adequate sensitivity make many of the currently available toxicity tests less desirable in situations such as monitoring human exposure to low concentrations of toxic chemicals routinely present in our environment or the workplace.

2 SYSTEMS TOXICOLOGY

Upon entering the biological system, toxic chemicals may interact with various cellular components and result in perturbation of the cellular processes vital for normal functioning of the system. The biological system, in turn, may respond to the chemical-induced perturbation of cellular processes by activating the cellular response system. It has been proposed that the balance between the chemical-induced perturbation of the biological system and the system's response to the perturbation will determine whether exposure to the chemical may eventually result in toxicity or not. It has been hypothesized that alterations in the global gene expression profile may precede the toxicity induced by chemicals in a biological system. Therefore, determination of the differential gene expression profile of a biological system that has been exposed to a toxic chemical may provide important information regarding not only the potential of the chemical to cause toxicity but also the mechanisms underlying the toxicity. The global gene expression profiling may provide a complete understanding of all the biological processes that are affected in a cell, a tissue, an organ, an organ system and all organ systems of an organism in response to exposure to a toxic chemical. Such an understanding is essential

to develop an ideal toxicity test capable of predicting/determining the potential of a chemical to cause toxicity as well as to elucidate the mechanisms underlying the toxicity. Recent advances in genomics and bioinformatics and their application in toxicology have facilitated the development of a new toxicology research area commonly referred to as toxicogenomics. Toxicogenomics is promising with respect to obtaining a complete understanding of the response of an organism to exposure to a toxic chemical. The three main areas of toxicogenomics are transcriptomics, proteomics and metabonomics. Toxicogenomics represents the complete understanding of the response at the level of transcripts (transcriptomics), proteins (proteomics) and metabolites (metabonomics). Integration of the cellular profiles of transcripts, proteins and metabolites with various traditional toxicological endpoints may provide a comprehensive view of the response of a biological system to exposure to toxic chemicals and this has emerged as a new toxicology branch commonly referred to as systems toxicology.

Systems toxicology may be defined as the understanding of all toxicological interactions taking place in a biological system under stress (chemical exposure). Thus, all these interactions between the exposure of the system to the toxic chemical and the ultimate elimination of the toxic chemical and/or its metabolites from the system may be considered as various aspects of systems toxicology. These include:

1. absorption and distribution of the chemical within the biological system;
2. metabolism of the chemical taking place in the biological system resulting in the generation of toxic and/or non-toxic metabolites;
3. interaction of the toxic chemical and/or its toxic metabolites with the cellular target(s) of toxicity within the biological system;
4. alterations in the expression profile of genes (transcripts) that may be related to the toxicity of the chemical;
5. alterations in the expression profile of proteins that may be related to the toxicity of the chemical;
6. histological changes in target organs that may be considered as structural manifestations of toxicity;
7. alterations in the biochemical constituents such as the release of enzymes from target organs into the bloodstream, which may be considered as functional and/or structural manifestations of toxicity;
8. response of the biological system such as the activation of cellular defence system to defend against the toxicity of the chemical; and
9. elimination of the chemical and/or its metabolites from the biological system.

The knowledge obtained from systems toxicology has implications in various aspects of chemical toxicity such as:

1. assessing the risk of toxicity and adverse health effects following exposure to potentially toxic chemicals present in the environment and workplace;
2. determining the safety and toxicity of drug molecules;
3. developing biomarkers to monitor human exposure to potentially toxic chemicals;
4. determining the mechanism(s) underlying the toxicity of chemicals; and
5. developing strategies to prevent the toxicity of chemicals.

As the objective of systems toxicology is to obtain a complete understanding of the response of a biological system to exposure to a toxic chemical, toxicogenomics (transcriptomics, proteomics and metabonomics) seems to have the potential to provide the data required for such an understanding. Of the three areas of toxicogenomics, transcriptomics has enjoyed the most significant growth to date, and therefore the impact of transcriptomics will be further discussed in this chapter.

3 TRANSCRIPTOMICS – TECHNICAL ASPECTS

Transcriptomics or transcript profiling deals with determination of the abundance of all transcripts (mRNA) that are expressed in a cell at a given time. Transcriptomics may be considered as a snapshot of all the expressed transcripts in a biological sample at a given time. It is assumed that exposure of cells to toxic chemicals results in a biological response, and a full understanding of the cellular response to the chemical exposure is important

in predicting, determining and understanding the toxicity of the chemical. This can be achieved by determining the abundance of all expressed genes (transcripts) in the chemical-exposed sample and comparing it with that of a control, unexposed sample. Several techniques are currently available to determine the mRNA expression profile of cells, including northern hybridization, PCR amplification, subtractive hybridization, serial analysis of gene expression, differential display and microarray analysis. Microarray analysis has the unique advantage of simultaneously determining the expression profile of thousands of genes present in biological samples and has been the method of choice to determine the global gene expression profile in biological samples. Microarray analysis of global gene expression profile is a complex process consisting of several sequential steps. The first step in the microarray analysis of gene expression profilings is the isolation of high-quality total RNA (it is not necessary to purify the mRNA) from the control and chemical-exposed biological samples. Subsequently, the mRNA is reverse transcribed to synthesize cDNAs. The cRNAs synthesized from the cDNAs are labelled with fluorescent dye, for example cy3, and the resulting targets are hybridized to the probes which are arrayed on the microarray. The sequence-specific hybridization of the labelled targets with the probes present on the microarray is subsequently detected by scanning the arrays with a fluorescence scanner and the intensity of the hybridization signal obtained is considered as an indication of the abundance of the targets (transcripts) expressed in the sample. The resulting data can be analysed using appropriate computational and statistical programs to determine the transcript profile of the sample. Comparison of the transcript profile of the chemically treated sample with the appropriate control sample will provide information about transcripts that are differentially expressed as a result of the chemical exposure. Appropriate bioinformatic analysis of the differentially expressed genes, integration of the differential gene expression profile with traditional toxicity endpoints and careful interpretation of the resulting data in light of the existing toxicology literature should provide a complete understanding of the events taking place in the biological system in response to exposure to the toxic chemical and in turn may contribute to systems toxicology (Figure 2).

Transcriptomics research has enjoyed considerable growth over the past several years. The discovery of microarray technology and its impact on transcriptomics studies have revolutionized toxicology research. The amount of RNA required for microarray studies has decreased significantly over the years and this has facilitated the use of this powerful technique even in difficult-to-obtain precious samples such as human tissues. Microarrays and the reagents and equipments required for analysis of global gene expression profiles are currently available from several manufacturers, and this has resulted in a significant reduction in the cost associated with the analysis. Currently, the microarray-based transcriptomics profiling technique has become an affordable technique that can yield reliable, reproducible and biologically relevant toxicity data. The impact of transcriptomics in toxicology research is illustrated by the number of research papers published in recent years that describe gene expression data (Figure 3). To date, microarray-based transcriptomics studies have been conducted in a wide variety of species that include nematodes (Menzel *et al.*, 2009), fish (Baker *et al.*, 2009), amphibians (Langerveld *et al.*, 2009), rodents (Courter *et al.*, 2008; Xu *et al.*, 2008), birds (Gust *et al.*, 2009), dogs (Higgins *et al.*, 2003), non-human primates (Marvanova *et al.*, 2003) and humans (McHale *et al.*, 2007). Transcriptomics-based toxicogenomics studies have been employed to study the toxicity of chemicals targeting various organs including brain (Glover *et al.*, 2009), eye (Yang *et al.*, 2007), nose (Roberts, Thomas and Dorman, 2008), lungs (Rouse *et al.*, 2008), heart (Carney *et al.*, 2006), kidney (Amin *et al.*, 2004), reproductive organs (Fukushima *et al.*, 2005; Valdez *et al.*, 2009), immune system (Baker *et al.*, 2009) and skin (Gerecke *et al.*, 2009). However, most of the transcriptomics studies reported so far have involved chemicals that are primarily hepatotoxic in nature or target liver as the primary target organ (Reilly *et al.*, 2001; Uehara *et al.*, 2008;Blomme, Yang and Waring, 2009; Sano *et al.*, 2009). This is mainly because liver is the primary organ responsible for metabolizing toxic chemicals (Lee, 2003), and furthermore, liver toxicity has been cited as a major reason for withdrawal of new drugs from the market (Barros and Martin, 2008). Studies investigating hepatotoxicity of chemicals will be discussed in detail to illustrate the potential application of transcriptomics in studying the systems toxicity of chemicals.

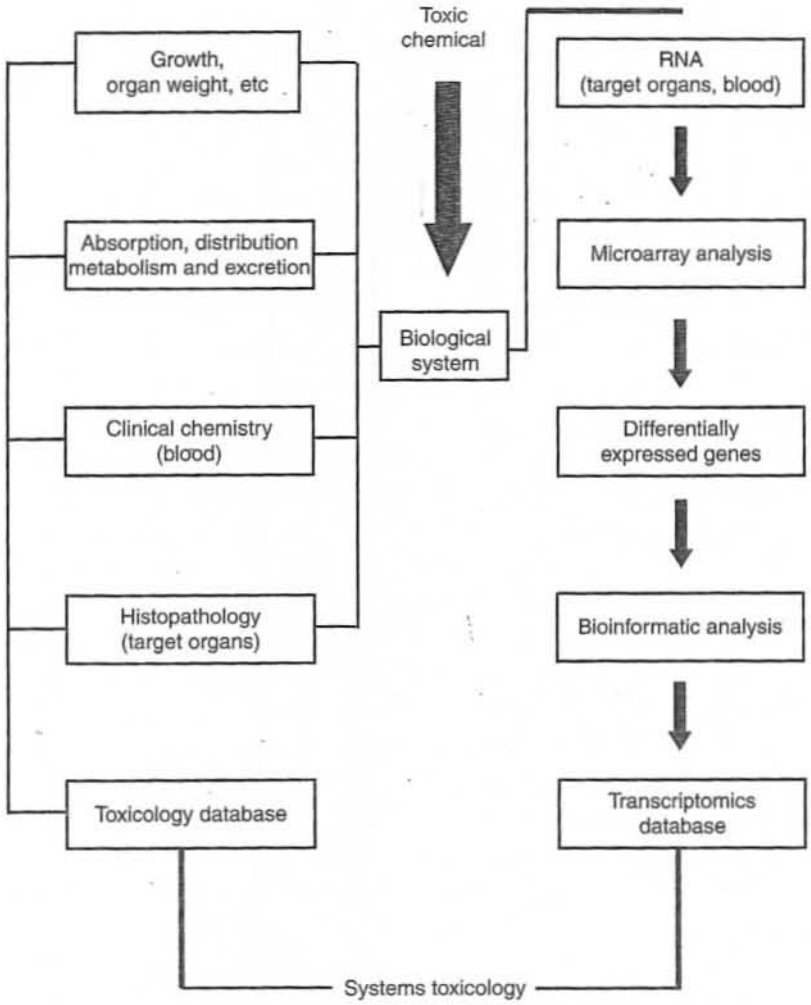

Figure 2. Integration of transcriptomics with traditional toxicity endpoints.

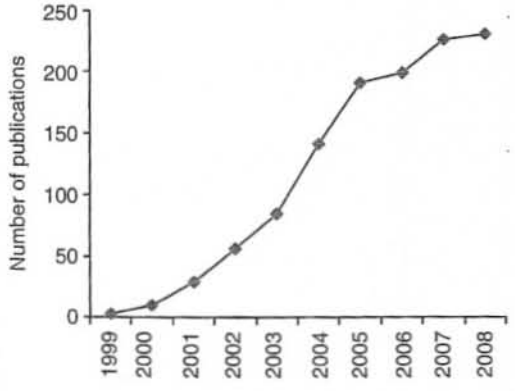

Figure 3. Impact of transcriptomics data in toxicology. The Pubmed database was queried using the keywords "microarray" and "toxicology," and the number of publications from 1999 to 2008 is presented.

4 HEPATOTOXICITY DETERMINATION BY TRANSCRIPTOMICS

The application of microarray-based transcriptomics studies in systems toxicity is best illustrated in the case of chemicals that target liver as their primary target organ. Hepatotoxic chemicals have been employed as model chemicals by several investigators to demonstrate the potential application of microarray-based global gene expression profiling to study the toxicity of chemicals (Bulera *et al.*, 2001; Hamadeh *et al.*, 2002a). So far, microarray-based transcriptomics studies have been conducted to classify hepatotoxic chemicals, determine mechanisms of hepatotoxicity and predict hepatotoxicity.

In a study conducted earlier by Waring *et al.* (2001), rats were treated with 15 different hepatotoxic chemicals including allyl alcohol, amiodarone, Aroclor 1254, arsenic, carbamazepine, carbon tetrachloride, dimethylnitrosamine, dimethylformamide, diquat, etoposide, indomethacin, methapyrilene, methotrexate, monocrotaline and 3-methylcholanthrene. The various chemicals selected in this study are all known to result in hepatotoxicity such as necrosis, DNA damage, cirrhosis, hypertrophy and hepatic carcinoma. Hepatotoxicity was determined by histopathology of liver and clinical chemistry evaluation of blood. In addition, global gene expression profiles of liver were determined by microarray analysis. Overall, a good correlation was noticed between histopathology, clinical chemistry and gene expression profiles induced by the hepatotoxic agents employed. The authors also attempted to cluster the various hepatotoxic compounds employed in the study based on their hepatotoxicity (histopathology and clinical chemistry results) and liver gene expression profiles. The results demonstrated that various hepatotoxic compounds employed in the study clustered together in fairly good agreement based on the observed changes in clinical chemistry, histopathology and gene expression profile. In some cases, liver gene expression profiles outperformed the traditional hepatotoxicity endpoints (histopathology of liver and blood clinical chemistry) in clustering the hepatotoxic chemicals. The authors, based on the results of their study, concluded that microarray-based global gene expression profiling could be employed as a highly sensitive and reliable technique to identify hepatotoxic chemicals in rat.

Whether gene expression profiling can be employed to distinguish (classify) hepatotoxic chemicals that differ in their mechanism of toxicity has been investigated (Hamadeh *et al.*, 2002b). Hepatotoxicity was induced in rats by administering either peroxisome proliferators (clofibrate, Wyeth 14 643 and gemfibrozil) or an enzyme inducer (phenobarbital). Hepatotoxicity and liver gene expression profiles of the rats were determined by histopathology and microarray analysis, respectively. The microarray data were subjected to several computational analyses to determine whether hepatotoxic chemicals exhibit gene expression patterns distinguishable based on their mechanism of toxicity. The results of this study demonstrated that the peroxisome proliferators exhibited a gene expression pattern that was clearly distinguishable from that of the enzyme inducer, suggesting the potential application of microarray-based gene expression profiling to classify hepatotoxic chemicals based on their mechanism(s) of toxicity.

A major advantage of gene expression profiling in toxicity studies is its superior sensitivity to detect target organ toxicity compared with the traditional toxicity endpoints such as histology and clinical chemistry. Several studies have indicated that gene expression changes indicative of hepatotoxicity are detectable well before the onset of clinical or histopathological changes associated with hepatotoxicity. In a study conducted by Heinloth *et al.* (2004), rats were administered either sub-toxic (50 and 150 mg/kg b.w.) or toxic (1500 mg/kg b.w.) doses of acetaminophen. At time intervals of 6, 24 and 48 h following administration of the chemical, groups of rats were killed and the induction of hepatotoxicity was determined on the basis of histopathology of liver and clinical chemistry of blood. At each time interval, RNA was isolated from the liver and global gene expression profile was determined by microarray analysis. At all time intervals, the rats administered the sub-toxic doses of acetaminophen exhibited normal histology and clinical chemistry, suggesting either the absence of hepatotoxicity in the rats or the inability of these traditional hepatotoxicity endpoints, because of their poor sensitivity, to detect subtle toxicity induced by the chemical. Similarly, rats administered the overtly toxic dose of acetaminophen (1500 mg/kg b.w.) did not exhibit alterations in either histology or clinical chemistry at the earliest time interval of 6 h following administration of the chemical. However, significant hepatotoxicity, as indicated by alterations in liver histology and blood clinical chemistry, was noticed in rats administered 1500 mg acetaminophen/kg b.w. at later time intervals of 24 and 48 h. Microarray analysis of global gene expression profiling demonstrated significant differential expression of several genes including those involved in energy metabolism and stress response in the liver of the rats administered 1500 mg acetaminophen/kg b.w. for 24 and 48 h. Interestingly, many of the genes which were found differentially expressed at time intervals of 24 and 48 h in the rats administered the overtly toxic dose of 1500 mg/kg b.w., and are therefore considered as indicators of acetaminophen-induced hepatotoxicity, were also

found differentially expressed in the rats administered the sub-toxic doses of the chemical. It is worth emphasizing that the rats administered the sub-toxic doses of acetaminophen did not exhibit any histological or clinical changes indicative of toxicity. Taken together, these results demonstrated the superior sensitivity of gene expression changes to detect hepatotoxicity compared with the traditional histological and clinical markers of toxicity. Similar observations supporting the superior sensitivity of gene expression profiling compared with the traditional hepatotoxicity endpoints in detecting hepatotoxicity have been reported by other investigators (Hamadeh *et al.*, 2002b).

The results of a recent study carried out by Huang *et al.* (2008) further support the findings that gene expression profiling can be employed to predict hepatotoxicity as well as understand the mechanisms of chemical toxicity in the liver. Rats were administered individually one of the seven hepatotoxic chemicals that are known to induce necrosis. Microarray analysis of the global gene expression profile in the rats identified several differentially expressed genes in the chemical-treated rats compared with the controls. Using a Random Forrest classifier with feature selection, a group of 21 genes were identified as predictors of necrosis induced by the hepatotoxic chemicals. The selected predictor genes were able to predict necrosis induced by the model hepatotoxic chemicals such as acetaminophen, carbon tetrachloride and allyl alcohol with an accuracy of 90%, 80% and 60%, respectively. Pathway and network analysis of the necrosis predictor genes identified inflammation and apoptosis as major biological processes responsible for necrosis induced by the selected hepatotoxic chemicals.

5 TRANSCRIPTOMICS STUDIES USING SURROGATE TISSUES

Toxicogenomics is certainly a viable and attractive approach to study the systems toxicity of chemicals in humans. However, a major limitation in studying the toxicity of chemicals in human beings is the difficulty associated with obtaining samples of target organs to study. Because of ethical and/or other practical issues such as the health and safety of the individuals involved, it is not possible to obtain samples of target organs or tissues from human beings to determine chemical toxicity. A practical solution to this problem is the potential use of surrogate tissues to study the toxicity of chemicals in target organs that are not easily and safely obtainable. Some surrogate tissues most commonly employed to study target organ toxicity are blood, urine, saliva, sweat, tear, cerebrospinal fluid, milk, broncho-alveolar lavage, sputum, stool, breath condensate, nail, hair, buccal cells and placenta (Burczynski and Rockett, 2006). From the surrogate tissues listed above, it is clear that many of them are not actually tissues in a biological sense. Any biospecimen that can be used to determine or study the biological processes taking place in target organs that are not easily obtainable can be considered a surrogate tissue. Of all the surrogate tissues employed to study target organ toxicity, blood is not only the most frequently used but also the most suitable one (Burczynski, and Rockett 2006). Blood may be considered as the internal environment for the various organs and tissues in the body that are often the targets for chemical toxicity. Very often, alterations in the biological processes taking place in the target organs and tissues in response to their exposure to toxic chemicals are reflected by alterations that are similar to a certain extent in the blood. Alterations in the activity of specific enzymes or the absolute amount of certain biochemical molecules or even the amount of the parent compound and/or its metabolite(s) present in the circulating blood or blood cells may provide valuable information regarding the target organ toxicity potential of chemicals; therefore, one or more of these indicators may be used as surrogate markers of toxicity. Activities of transaminases (aspartate and alanine aminotransferase) in the blood are routinely employed as reliable surrogate markers of hepatotoxicity (Heinloth *et al.*, 2004; Beyer *et al.*, 2007; Bushel *et al.*, 2007). Other surrogate markers of toxicity are blood urea nitrogen (BUN) for nephrotoxicity (Fang *et al.*, 2008; Roomi *et al.*, 2008), serum acetyl cholinesterase activity for neurotoxicity induced by organophosphorus insecticides (Brahmi *et al.*, 2006), blood level of hormones for toxic chemicals targeting endocrine system (De Angelis *et al.*, 2009) and so on.

Although these surrogate toxicity markers have been of immense help to determine the target organ toxicity of chemicals, all or most of them suffer from significant shortcomings such as not being specific for the target organ(s) involved or not being sensitive enough to detect toxicity in a pre-clinical

condition. For example, significant structural damage, such as necrosis, to the hepatocytes is necessary for transaminases to be released from the liver into the peripheral blood and then measured as markers of hepatotoxicity. Furthermore, an elevation in the serum level of transaminases need not always be an indicator of chemical toxicity taking place in the liver. For example, significant elevations in serum transaminases have been reported under conditions other than exposure to hepatotoxic chemicals (Harrison, Bahar and Payne, 2002; Ramakers *et al.*, 2009).

As presented earlier in this chapter, gene expression changes taking place in target organs are known for their superior sensitivity as markers of toxicity compared with the traditional biochemical and histological markers of toxicity. Therefore, it seems reasonable to investigate whether gene expression profiling to determine target organ toxicity can be performed using easily available surrogate tissues such as blood. This concept has recently gained popularity, and there have been few studies reporting the potential application of blood gene expression profiling as sensitive surrogate indicators of target organ toxicity. As in the case of target organs, most of the toxicogenomics studies investigating the potential application of blood transcriptomics as surrogate markers of target organ toxicity have been conducted using model hepatotoxic chemicals. In a classical study, Bushel *et al.* (2007) of the National Institute for Environmental Health Sciences (NIEHS) demonstrated the usefulness of blood transcriptomics as sensitive indicators of hepatotoxicity induced by the model hepatotoxic drug acetaminophen in rats. Rats were given either a subtoxic (150 mg/kg b.w.) or two overtly toxic (1500 and 2000 mg/kg b.w.) doses of acetaminophen. Blood and liver obtained from the control and acetaminophen-administered rats were analysed to determine hepatotoxicity induced by the chemical. The parameters employed included liver histology, various haematological parameters (total and differential white blood cell counts, red blood cell count, platelet count, hematocrit and haemoglobin content), blood level of transaminases and global gene expression profiling in blood. Based on the results obtained, these authors demonstrated that blood gene expression profiles may be employed as surrogate markers of hepatotoxicity induced by acetaminophen. Furthermore, the results demonstrated that blood gene expression profiles exhibited superior sensitivity as hepatotoxicity markers compared with the traditional toxicity endpoints such as histological, haematological and biochemical changes.

The potential application of blood transcriptomics as appropriate surrogate marker of target organ toxicity was further demonstrated by the results of a comprehensive study published recently (Lobenhofer *et al.*, 2008). These investigators employed a series of eight well-characterized hepatotoxic chemicals that are known to exhibit similarities and differences in the type and location of necrosis induced in the target organ (liver) to demonstrate the potential use of blood transcriptomics as surrogate marker of hepatotoxicity. The study was designed to provide a means to generate mechanistic and predictive measures of toxicity by integrating multiple data streams obtained from liver (target organ) and blood (surrogate tissue). The hepatotoxic chemicals selected were administered in rats at three doses (low, medium and high) for three time intervals (6, 24 and 48 h) to result in sub-toxic to severe hepatotoxicity (necrosis). Toxicity was determined based on established histological, biochemical and haematological parameters in liver (the target organ) and blood (surrogate tissue). In addition, gene expression profiling of liver and blood was performed using two separate microarray platforms. A support vector machine (SVM) approach was employed to identify hepatotoxicity-specific classifiers for the target organ and the surrogate tissue. The classifiers thus obtained were used to group the samples based on principal component analysis and hierarchical clustering to determine whether the animals exposed to each of the hepatotoxicants could be separated into different compound groups. Results of the study demonstrated that the classifiers derived from both liver and blood transcriptomic data were able to accurately classify samples on the basis of the hepatotoxic chemical administered. It is noteworthy to mention that the classifiers generated from the blood data outperformed those generated from the liver data in classifying the hepatotoxicants.

Results of recent studies carried out in our laboratory (Umbright *et al.*, 2009) further support the concept that blood gene expression profiling may be employed as a suitable, alternate approach to detect and distinguish target organ toxicity induced by toxic chemicals. Rats were administered a single, acute dose of a well-characterized hepatotoxic (acetaminophen) or a

neurotoxic (methyl parathion) chemical. Administration of acetaminophen resulted in overt hepatotoxicity as evidenced from significant elevation in blood transaminases' activities. Similarly, administration of methyl parathion resulted in neurotoxicity as evidenced from significant inhibition of acetylcholinesterase activity. Microarray analysis of the global gene expression profile of leucocytes obtained from the blood samples of rats identified distinct gene expression profiles capable of detecting and distinguishing hepatotoxicity and neurotoxicity induced by the model chemicals administered. The gene expression markers of target organ toxicity were detectable in the blood earlier than the appearance of the commonly used clinical markers of toxicity, further supporting the superior sensitivity of gene expression markers compared with traditional toxicity endpoints to detect target organ toxicity (Figure 4). The ability of the marker genes to detect and distinguish hepatotoxicity and neurotoxicity was further confirmed using the blood samples of rats administered additional model hepatotoxic (thioacetamide, carbon tetrachloride and dimethylnitrosamine) and neurotoxic (ethyl parathion and malathion) chemicals.

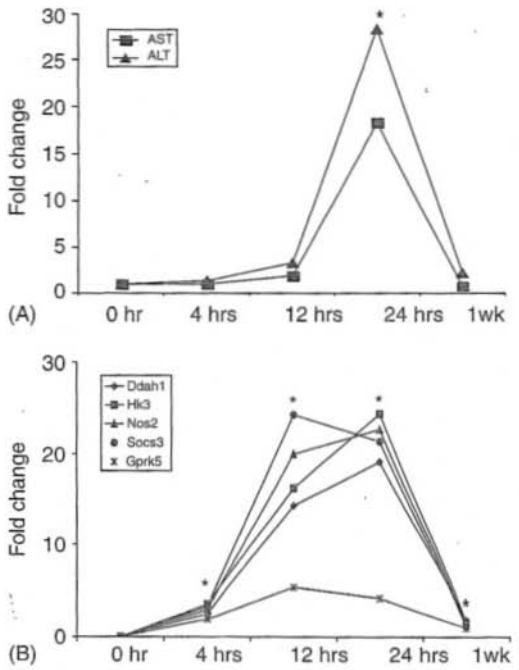

Figure 4. Gene expression profiling is more sensitive than the traditional toxicity endpoints. A single acute toxic dose of acetaminophen was administered in rats. At various time intervals ranging from 4 h to 1 week following administration of the chemical, blood was analysed for toxicity based on the activities of AST and ALT (a) and expression of marker genes for hepatotoxicity (b). Significant alterations in the expression of the selected hepatotoxicity marker genes were observed in the blood before any significant change in the activities of transaminases, suggesting the superior sensitivity of gene expression changes as indicators of target organ toxicity (Reproduced with permission from Umbright *et al.* (2009)).

6 TRANSCRIPTOMICS STUDIES – CHALLENGES

The past 10–15 years have witnessed significant progress in the application of transcriptomics in studying the toxicity of chemicals. Nevertheless, microarray-based transcriptomics research still faces many challenges, and further attention to these issues or challenges is required to enhance the potential of transcriptomics in toxicogenomics and systems toxicology. Following is a description of some of the major challenges facing transcriptomics and the progress that has been achieved.

6.1 Confirmation of Microarray Data

In spite of the high-throughput potential, transcriptomics data are usually error-prone. It is therefore possible to notice several false positives and false negatives among the hundreds if not thousands of differentially expressed genes that are identified in a chemically treated sample based only on microarray determination of gene expression profiles. It is often necessary to confirm the microarray data to eliminate at least false positives as much as possible. Appropriate statistical methods to minimize the false discovery rate (FDR) are usually applied while analysing the microarray data (Benjamini and Hochberg, 1995). Another approach is to confirm the microarray data by more quantitatively reliable supplementary techniques such as real-time PCR or northern hybridization. However, considering the hundreds if not thousands of differentially expressed genes that may be identified from a typical microarray experiment, it is a challenging task to confirm the microarray data by these cumbersome supplementary techniques. It is worthy to mention that microarray technology has improved considerably since its introduction in the mid-1990s and it has

become a more reproducible and reliable technique capable of detecting differentially expressed genes with significant accuracy. A reasonably good correlation has been noticed in the gene expression profile data obtained by microarray and real-time PCR analysis (Joseph, He and Umbright, 2008; Umbright *et al.*, 2009).

6.2 Comparability of Transcriptomics Data

A major roadblock in microarray-based transcriptomics research is the comparability of expression profiling data obtained from multiple sources (Irizarry *et al.*, 2005; Larkin *et al.*, 2005). As described earlier in this chapter, microarray analysis of gene expression profiling is quite complex and technologically challenging. Each step in the analysis can potentially contribute to variability in the data. Depending on the microarray platforms employed (one channel vs. two channels, oligonucleotide vs. cDNA, in-house fabricated vs. commercially available), the same biological sample may yield different gene expression profiles. It is worth mentioning that significant refinement in microarray technology has taken place since its introduction in the mid-1990s and this has facilitated reasonably good concordance in microarray data obtained using different microarray platforms (Liew *et al.*,2006). Each step in the microarray analysis procedure, such as RNA isolation, reverse transcription and synthesis of labelled cRNA targets, hybridization and washing of chips, and acquisition and analysis of data, can contribute to variations in microarray data. Variability in the microarray data generated by different investigators can have implications in the ultimate utility of the microarray data generated. Therefore, it is necessary to take precautions or even to develop standards for conducting microarray experiments so as to minimize the variability associated with the technical aspects of microarray experiments. Similar to the guidelines recommended by MIAME (minimum information about microarray experiment) for documentation of microarray data (Brazma *et al.*, 2001), standards for conducting microarray experiments may be developed to minimize the variability associated with the microarray data generated in different laboratories.

Several studies have been conducted in the past to address the variability or comparability issue of microarray data. The Toxicogenomics Research Consortium (Bammler *et al.*, 2005) has identified some of the potential sources of variability in microarray data. In a study conducted by investigators of the Toxicogenomics Research Consortium, microarray data were generated by seven participating laboratories using two standard RNA samples and 12 microarray platforms. It was noticed that reproducibility for most microarray platforms was generally good when the data were generated by the same participating laboratory. Most of the variations were noticed when the data were generated by different laboratories. Reproducibility among the participating laboratories increased markedly when standardized procedures were implemented for RNA isolation, target labelling, microarray hybridization, data acquisition and processing. It was also noticed that the variability was least when the analysis was performed based on biological themes.

The contribution of biological variability in addition to technical issues associated with the quality of microarray data has been demonstrated by the results of a study conducted by the investigators from seven geographically dispersed research centres in the USA (Beyer *et al.*, 2007). Several precautions were taken in an attempt to avoid, or at least minimize, the variations in the data generated by the participating laboratories. The study involved microarray analysis of the gene expression profile of the liver of mice administered a toxic dose of acetaminophen. Even though administration of acetaminophen in mice was conducted independently in the participating laboratories, they all received mice, feed, bedding and acetaminophen from centralized sources. Furthermore, all participating laboratories were required to adhere to a standard protocol for treatment, treatment termination, specimen collection and processing. The specimens collected in the individual laboratories were sent to a centralized facility for measurement of endpoints [liver histology, serum alanine aminotransaminase (ALT) activity and liver gene expression profile]. Administration of acetaminophen, in general, resulted in hepatotoxicity as evidenced from histological changes (necrosis) in the liver and elevation of ALT activity in the blood of the mice. However, animal-to-animal variability was noticed with respect to the liver toxicity induced by acetaminophen, and this was more

prominent among the laboratories than within an individual laboratory. A corresponding variation in the number of significantly differentially expressed genes was noticed among the laboratories. The variability observed in gene expression profiles was significantly reduced when the differential gene expression data were anchored to the liver injury phenotype (the importance of anchoring differential gene expression profiles to toxicity phenotype is further discussed elsewhere in the chapter).

The Carcinogenicity Working Group of the Predictive Safety Testing Consortium, a collaboration between several pharmaceutical industries and the US Food and Drug Administration (FDA), has tested (Fielden *et al.*, 2008) two published hepatic gene expression signatures (Nie *et al.*, 2006;Fielden, Brennan and Gollub, 2007) developed to predict carcinogenic potential of chemicals in rats. The microarray data obtained from two short-term rat studies using over 150 compounds (carcinogenic and non-carcinogenic) were subjected to a meta-analysis for predicting carcinogenicity. It was found that despite significant differences in the study designs and microarray platforms employed in obtaining the data, the signatures proved to be relatively robust and more accurate than expected by chance (Fielden *et al.*,2008). However, a considerable loss (15–30%) in accuracy of the predictive signature was noticed when the signature generated by the individual laboratories was further validated. A loss of 15–30% accuracy is substantial and could result in false classification of a potentially carcinogenic chemical as non-carcinogenic or vice versa. The loss in accuracy of predictability was attributed mainly to the differences in experimental conditions, treatment protocols and microarray platforms employed.

The studies presented above unequivocally support the observation that variability is an integral part of microarray data. Furthermore, they have identified some of the potential sources such as technical issues and biological variation that are responsible for the variability observed. Standardization of procedures employed to obtain the microarray data and incorporation of biological themes (phenotypic anchoring) when analyzing the microarray data can significantly reduce variability in the microarray data generated by different investigators. Such improvements can result in more reliable and toxicologically relevant microarray data that can be used to determine the toxicity of chemicals including those to which significant human exposure may take place.

6.3 Biological Relevance of Transcriptomics Data

Another major challenge facing transcriptomics-based systems toxicology is determining the toxicological relevance of the transcriptomics data. Typically, microarray analysis of gene expression profiling may result in the identification of hundreds or even thousands of genes that are significantly differentially expressed in a biological system in response to its exposure to a toxic chemical. Some of these changes may be detected by chance and as such may have no toxicological relevance, whereas others may be related to the chemical-induced perturbation of the biological system and therefore are expected to be toxicologically relevant. What would be the criteria to determine whether a certain gene expression profile is toxicologically relevant or not? Toxicology research conducted over the past several decades has identified several cellular events that are considered reliable endpoints of toxicity. For example, exposure of a rat to hepatotoxic chemicals results in liver damage that can be detected by histological analysis of the tissue. The damaged liver cells in turn release enzymes, including transaminases, (aspartate and alanine transaminases) into the bloodstream, resulting in elevated serum levels of these enzymes. In general, a good correlation has been noticed between the serum level of transaminases and the liver damage or toxicity induced by hepatotoxic chemicals. In a similar way, it is possible to establish a relationship between differentially expressed genes and the established histological or biochemical markers of toxicity. This is commonly referred to as phenotypic anchoring (Paules, 2003; Powell *et al.*, 2006). Phenotypic anchoring is defined as establishing a correlation or relationship between the toxicogenomics (transcriptomics, proteomics and metabonomics) data and the toxicity phenotype as determined by traditional toxicity endpoints. As many experimental details as possible are required for the successful anchoring of a gene expression profile to a toxicity phenotype. For example, information regarding the dose of the chemical administered may suggest whether a

particular gene expression profile is an indicator of subtle toxicity or overt toxicity. Similarly, the time elapsed between exposure to the chemical and the determination of the expression profile may suggest whether a certain profile is a marker for early or late toxicity.

The importance of phenotypic anchoring in understanding the toxicological relevance of gene expression data is best illustrated in the case of hepatotoxicity. In a study by Heinloth *et al.* (2004), the prototypical hepatotoxic drug, acetaminophen, was administered in rats at doses of 0, 50, 150 and 1500 mg/kg b.w.. The control and the drug-administered rats were sacrificed at time intervals of 6, 24 and 48 h following administration of the chemical. Administration of acetaminophen at the selected doses and durations resulted in different grades of toxicity ranging from no toxicity (lower doses and early time points) to severe toxicity (higher doses and late time intervals) as revealed by the results of liver histopathology and blood clinical chemistry. Microarray analysis of the global gene expression profile in the liver resulted in the identification of a large number of differentially expressed genes in the acetaminophen-administered rats compared with the controls. Hierarchical clustering of the differentially expressed genes at any dose or time showed that there were clusters of genes similarly regulated at all three doses of the chemical, including the lower doses that did not result in any detectable change in liver histology or blood clinical chemistry. Bioinformatics analysis of the differentially expressed genes revealed that genes involved in energy-producing biochemical pathways were up-regulated whereas those involved in energy-consuming pathways were down-regulated in the acetaminophen-administered rats including those administered the lowest, non-toxic dose of the chemical. Biochemical analysis revealed significantly reduced ATP levels in the livers of the acetaminophen-administered rats compared with the controls, further supporting the microarray data. Similarly, Powell *et al.* (2006) have successfully anchored gene expression signatures in acetaminophen-administered rats to conventional biomarkers of oxidative stress. Thus, phenotypic anchoring has demonstrated the capability of a gene expression profile to detect and predict toxicity as well as to determine the mechanisms underlying the toxicity of chemicals. In addition, as discussed above, phenotypic anchoring of differentially expressed genes also demonstrated the superior sensitivity of gene expression profiles as indicators of toxicity compared with traditional toxicity endpoints.

6.4 Establishing Standards in Acquiring and Processing Transcriptomics Data

As described above, a major challenge facing transcriptomics-based toxicogenomics research is the difficulty in comparing data generated by different investigators. Although the contribution of factors such as biological variability of experimental samples in this regard is unavoidable, there are a number of factors, especially those related to acquiring and processing the transcriptomics data, that can be controlled so as to minimize the differences in the transcriptomics data generated by various investigators. For example, standardization of techniques such as isolation of RNA from biological samples, synthesis of labelled targets, microarray hybridization and washing, and microarray scanning to obtain gene expression signals may minimize the technical variability associated with transcriptomics data, facilitating better comparability of microarray data among investigators. Similarly, standardization of data analysis procedures may enhance the comparability of transcriptomics data obtained from different laboratories. Different investigators, for example, have adopted different criteria to select significantly differentially expressed genes. Although some investigators have applied correction for FDR (Bushel *et al.*, 2007; Huang *et al.*, 2008), others have not (Reilly *et al.*, 2001; Minami *et al.*, 2005). Differentially expressed genes have been selected solely on the basis of fold-change in expression (Reilly *et al.*, 2001; McHale *et al.*, 2007) or *p*-value (Huang *et al.*, 2008). There are also cases where a combination of fold change in expression and statistical significance of the data has been employed to select significantly differentially expressed genes (McHale *et al.*, 2007; Elferink *et al.*, 2008; Umbright *et al.*,2009). Thus, depending on the selection criteria employed (fold change in expression, statistical significance or a combination of both), considerable differences in the genes selected as significantly differentially expressed must be expected. This, in turn, may result in corresponding differences in the results obtained from further downstream analysis

of gene expression data such as determination of the involvement of specific pathways, networks and biological functions in the toxicity of the chemical being investigated. Thus, adopting standards for acquiring and analysing microarray data may reduce the variability in the data generated by different investigators, facilitating better comparison of the transcriptomics data. It is encouraging to notice that the standardization efforts by various groups such as the Microarray Quality Control Consortium and the Toxicogenomics Resource Consortium have resulted in considerable improvements in the reproducibility of transcriptomics data (Bammler *et al.*, 2005; Irizarry *et al.*, 2005; Larkin *et al.*, 2005; Shi *et al.*, 2006; Beyer *et al.*, 2007).

6.5 Translation of Transcriptomics Data from Experimental Models to Humans

Most of the toxicogenomics studies reported so far have employed cell cultures or experimental animals as models to derive microarray data relevant to the toxicity of chemicals. The ultimate goal of any toxicity study employing experimental models is to derive data that are relevant and applicable to situations of human exposure to chemicals resulting in potential toxicity. The role of confounding factors potentially capable of influencing the gene expression profile is more significant in the case of humans compared with the experimental models. Experimental models such as animals are relatively homogeneous in nature and factors such as species, strain, diet, exposure to other chemicals, etc. can be controlled in them to minimize the confounding effects of these factors on gene expression profile. In contrast, individuals who may be exposed to toxic chemicals present in the environment or the workplace are very heterogeneous in nature and may be subjected to a large number of confounding factors such as race, age, sex, life style factors and exposure to other chemicals that may potentially influence the gene expression profile. It needs to be thoroughly investigated whether toxicogenomics data derived from well-controlled experiments involving cell culture and animal models can be translated to highly variable situations of human exposure to toxic chemicals. In a recent study published by Bushel *et al.* (2007), a rat experiment was conducted to identify a discriminatory blood gene expression profile for hepatotoxicity induced by acetaminophen. The investigators were able to identify acetaminophen-overdosed patients from the controls by employing human orthologues for the rat discriminatory genes they had identified. Although this study has demonstrated the potential translation of transcriptomics data derived from experimental animals to humans under conditions of exposure to relatively large doses of acetaminophen capable of causing overt liver toxicity, it is not known whether the same can be applied to situations of human exposure to low concentrations of toxic chemicals present in the environment as well as the workplace.

7 DISCLAIMER

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Handbook of Systems Toxicology

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