Review

Toxicogenomic Biomarkers for Liver Toxicity

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Abstract: Toxicogenomics (TGx) is a widely used technique in the preclinical stage of drug development to investigate the molecular mechanisms of toxicity. A number of candidate TGx biomarkers have now been identified and are utilized for both assessing and predicting toxicities. Further accumulation of novel TGx biomarkers will lead to more efficient, appropriate and cost effective drug risk assessment, reinforcing the paradigm of the conventional toxicology system with a more profound understanding of the molecular mechanisms of drug-induced toxicity. In this paper, we overview some practical strategies as well as obstacles for identifying and utilizing TGx biomarkers based on microarray analysis. Since clinical hepatotoxicity is one of the major causes of drug development attrition, the liver has been the best documented target organ for TGx studies to date, and we therefore focused on information from liver TGx studies. In this review, we summarize the current resources in the literature in regard to TGx studies of the liver, from which toxicologists could extract potential TGx biomarker gene sets for better hepatotoxicity risk assessment. (J Toxicol Pathol 2009; 22: 35–52)

Key words: toxicogenomics, biomarker, liver, microarray

Introduction

Although the term “toxicogenomics” (TGx) is relatively new, this method is now widely utilized by pharmaceutical scientists to investigate the molecular mechanisms of toxicity. Although the importance of functional genomics has been recognized since the emergence of microarray technology¹², more attention has been focused on it since the US Food and Drug Administration (FDA) released a whitepaper¹ showing that the number of new molecular entities has been decreasing since 2000, but that the costs of pharmaceutical companies for R&D of drugs have increased dramatically since 1993. One of the major attritions in the drug development process lies in unexpected adverse effects elicited in the clinical phase, and therefore the preclinical toxicological evaluation and the clinical trial steps are called ‘critical path’ of drug development in the FDA whitepaper. One estimation suggests that a 10% improvement in predicting future failure in the clinical phase would save 100 million dollars of R&D cost per drug¹, and the whitepaper emphasized the importance of modernizing toxicological methodologies by applying cutting-edge techniques such as TGx and other “-omics” techniques.

One of the goals of TGx research is to identify novel biomarkers for evaluating the efficacy or toxicity in either clinical or preclinical cases, which would be as useful as such conventional biomarkers such as the blood enzyme activity of alanine aminotransferase in evaluating liver injury. The term ‘biomarker’ is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or pharmacologic responses to a therapeutic intervention⁴. In principle, any biological parameters that are objectively measurable and recordable could be potential biomarkers. One example of a ‘good biomarker’ is single nucleotide polymorphisms (SNPs) in human CYP2C9 and Vitamin K epoxide reductase genes, which are used for optimization of the dosing level of warfarin, an anticoagulant drug with a great number of serious adverse effects in the US⁵. Such biomarkers are not only useful for efficient drug risk management but will also lead to the establishment of promising markets for pharmaceutical companies. In TGx research, the term ‘biomarker’ does not always refer to a single gene, but may consist of sets of genes whose expression levels are closely associated with certain toxicological endpoints.

In the TGx research field, the liver has been the preferred target organ for the following reasons: i) the clinical manifestation of hepatotoxicity is one of the major causes of drug development attrition; ii) the exposure level of the liver is exceptionally high following drug treatment; and iii) it is relatively easy to collect liver samples due to its size and homogeneity. In this paper, we outline the literature

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resources in regard to candidate TGx biomarkers for liver toxicity and overview their significance, and advantages and major obstacles in practical use.

**Microarray Technique**

Microarray is the most mature functional genomics technique and is now utilized in various fields, including pharmacology, toxicology and nutritional science. Compared with traditional gene expression analysis techniques such as Northern blotting or RT-PCR, microarray can measure the expression levels of tens of thousands of genes simultaneously, and accordingly, the data acquisition is considerably high-throughput (Table 1). In a microarray analysis, target samples (i.e., mRNA, cRNA or cDNA) are labeled with fluorescent dyes (i.e., Cy3, Cy5, phycoerythrin, etc.) of either one or two colors. The microarray probes consist of either cDNA or oligonucleotide and are hybridized with labeled target samples which have complementary nucleotide sequences.

Although microarrays can be manufactured in a lab using specific instruments, a number of microarray platforms are now commercially available, including GeneChip (Affymetrix, Inc.), Illumina (Illumina, Inc.), Codelink (GE Healthcare) and Agilent oligonucleotide arrays (Agilent Inc.). Each microarray platform has its own advantages and disadvantages. For example, in the Agilent 2-color (Cy3 and Cy5 dyes) microarray system, the Cy5 dye is extremely ozone-sensitive, and its signal is rapidly weakened under a high concentration of ozone, which results in extremely poor data quality. On the other hand, the Affymetrix GeneChip system requires specific instruments, and therefore the initial investment is quite high, while the cost of preparing a cDNA microarray in-house is relatively low, provided the cDNA clones and spotting instrument are available. Organizing and maintaining DNA clones, however, are tedious and error-prone procedures that can easily lead to confusion, and the reliability of the obtained data may sometimes be questionable. On the other hand, commercial microarrays usually provide specified kits that contain the entire reagent necessary for all the experimental processes and, in some cases, are even equipped with specialized instruments to automate tedious work such as washing and staining the microarrays after hybridization. Therefore, commercial microarrays are generally preferred by pharmaceutical researchers because they regard these advantages to be more cost-effective in the long term.

**Finding Differentially Expressed Genes**

Microarray fluorescence is detected with a scanner after washing the microarray after hybridization with labeled target samples. After scanning the microarray fluorescence signals, the scanned microarray image is subjected to gridding and assignment of predefined probe information using image analysis software such as GenePix Pro (Molecular Devices). Usually, this step is performed manually, and it is therefore a tedious procedure.

In the Affymetrix GeneChip system, this process is highly automated and easy to complete. After completion of the gridding, the image data with the fluorescent signals are converted into numerical data followed by background subtraction to correct any undesirable bias of the individual data derived from the experimental conditions, sample, manufacturing variability or other factors. A set of probes comprised of two types of probe per gene are designed in the GeneChip system, namely the Perfect Match (PM) and Mismatch (MM) probes (typically 11 MM and 11 PM probes that are 25-bp nucleotides in length) per gene. The PM probe sequence is complimentary to that of the target gene, while the MM probe sequence contains one mutated sequence in the middle of the 25 bp sequence, and the MM probe is used to estimate non-specific bindings to the PM sequence. Since multiple probes are designed for one gene, one needs to evaluate the expression level of the gene by summarizing multiple probe data sets. A number of analytical algorithms have been proposed for this “summarization” of the probe level data, including MAS5, dChip, RMA and GCRMA. MAS5 is a ‘chip-by-chip’ summarization algorithm, while dChip, RMA and GCRMA.

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**Table 1. Representative Analytical Methods for Gene Expression Studies**

| Method               | Sensitivity | Specificity | Throughput | Notes                                                                 |
|----------------------|-------------|-------------|------------|----------------------------------------------------------------------|
| Northern blot        | O           | O           | ×          | Size and alternatively-spliced variants of mRNA detectable           |
| RT-PCR               | O           | O           | △          | Cross hybridization of the used probe detectable                     |
| Real-time PCR        | O           | O           | △          | Need to optimize amplification cycle                                 |
| SYBR® Green          | O           | O           | △          | Easy to perform                                                      |
| TaqMan®              | O           | O           | △          | Relatively low cost                                                 |
| Microarray cDNA      | O           | ×           | O          | Moderately expensive                                                |
| Microarray Oligonucleotide array | O | O | O | Highly expensive                                                      |
| Microarray Expression array | O | O | O | Relatively high specificity                                          |
| Microarray Exon array | O           | O           | O          | Detect alternatively-spliced variants                                |

The meanings of the symbols in the table are as follows: ◎, Excellent; ◆, Very good; ◄, Good; ◄, Poor.
are ‘model-based’ or ‘project-based’ summarization algorithms that require relatively high performance computers to perform the calculations. In general, the project-based summarization algorithm yields better quality datasets in terms of sensitivity and reproducibility. On the other hand, MAS5 calculations are easy to compute, and there is no need to perform recalculations on whole data sets when new GeneChip data is added to a project. Thus, there is a trade-off in terms of the pros and cons of each method.

After correction of the individual data biases, the numerical data is subjected to normalization so that one can perform a comparative analysis among the microarray data sets. The easiest normalization is to adjust the global signal scale of each set of microarray data (global normalization), usually by setting it to the mean or median of the total signal data set. Another method is to use external spikes to get a standard curve, such as ‘Percellome normalization’8, in order to quantify the mRNA levels. This normalization method has been shown to be effective when the gene expression changes are extreme, such as in a uterotrophic response following activation of estrogen receptor or in an in vitro system using a primary cell culture.

After the normalization, one needs to identify the differentially expressed genes in the chemical-treated group. Since microarray analysis measures the expression levels of a large number of genes simultaneously, a straightforward pair-wise test, such as a t-test, would yield a considerable number of false-positives. (For example, if we set the significance level as \( P < 0.01 \) for Rat 230 2.0 GeneChip data consisting of \( > 30,000 \) probe sets, we may detect more than \( > 300 \) positives just by chance). To prevent this multiple testing problem, \( P \)-value correction may be performed using False Discovery Rate9, or two individual filtering criterions like fold change and \( t \)-value can be used in combination. A number of filtering methods are provided in the literature, such as significance analysis of microarrays (SAM)10, and there are a great number of sophisticated algorithms available as library files on the BioConductor project website (http://www.bioconductor.org/)11 that can be implemented via the open source statistical software R (http://www.R-project.org).

### Table 2. Representative Multivariate Analysis Methods

| Methods                        | Advantage                                                                 | Disadvantage                                               |
|--------------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------|
| **Unsupervised**               |                                                                           |                                                            |
| Hierarchical clustering        | Capture the trend of gene expression profiles easily without losing quantitative information | The output result is usually inconclusive and unclear         |
| K-means clustering             |                                                                           |                                                            |
| Self-organization map          |                                                                           |                                                            |
| Principal component analysis   | Easy to interpret the result because of highly reduced data dimension      | May lose significant information during reduction of data dimensions |
| **Supervised**                 |                                                                           |                                                            |
| K nearest neighbors (KNN)      | The output result is conclusive and clear                                  | Inappropriate training data set will generate a poor-performance discriminator |
| Support vector machine (SVM)   |                                                                           |                                                            |
| Prediction analysis of microarray (PAM) |                                                               |                                                            |

## Multivariate Analysis

Since microarray data consist of large amounts of numerical data, statistical knowledge, computational skills and infrastructure are required to interpret the results. Multivariate analysis methods are utilized for both data mining and pattern recognition (Table 2). “Unsupervised” multivariate analysis includes hierarchical clustering12, K-means clustering12, self-organizing map (SOM)12 and principal component analysis (PCA)13. “Supervised” multivariate analysis, or discriminant analysis, includes Support Vector Machine (SVM)14, K-Nearest Neighbors (KNN)15 and Prediction Analysis of Microarray (PAM)16. In general, each biomarker gene set requires its own specific analytical method based on the objective and manner of gene set identification.

Eisen et al. applied hierarchical clustering to visualize the trend of gene expression profiles17, and since then the hierarchical clustering method has been widely preferred by toxicologists when interpreting microarray data. In the case of K-means clustering and SOM, one needs to specify the number of clusters to be formed before the calculation. PCA is utilized to reduce the dimensions of the microarray data into 2 or 3; this makes it much easier to recognize the gene expression pattern.

Discriminant analysis, such as SVM, KNN and PAM, is an application of machine-learning algorithms and is frequently used for toxicity prediction based on microarray data. The sample size and appropriate selection of the training data set are crucial for establishing reliable classifiers. This type of discriminant analysis is also applied to quality control of microarray data18.

As described above, microarray analysis consists of multiple steps from in vivo / in vitro studies to microarray data interpretation (Fig. 1), and each step includes specific points to be considered in order to avoid misinterpretation of the obtained results.

## Literature Resources for TGx Biomarkers in Regard to Liver Toxicity

The reports in the literature related to liver toxicity-
relevant gene sets obtained from TGx studies are summarized in Table 3. A great number of TGx studies of the liver have been reported using various animal models, such as rats, mice, humans, monkeys and canines, and these studies contain a number of toxicity-relevant gene sets that could be potential TGx biomarkers for assessing/predicting liver toxicity.

Hepatotoxicity animal models using prototypical toxicants such as acetaminophen or carbon tetrachloride have been widely tested in TGx studies, and a number of gene sets associated with liver injury have been reported. Since these gene sets consist of a mixture of primary responses associated with cell death as well as secondary or more downstream responses such as inflammation caused by Kupffer cells or infiltrated lymphocytes, one needs to dissect the stimulated biological pathways carefully to interpret the biological significance associated with gene expression changes.

Waring et al. reported that the hepatic gene expression profiles in rats following treatments with various chemicals showed clear chemical-specific patterns. Based on this result, one can assume that such chemical-specific changes in the transcriptome profile would lead to changes in the proteome profile, the metabolome profile and eventually the histopathological phenotypes at later time points. This concept led toxicologists to expect that one might be able to utilize microarray data to predict later histopathological changes that are not detectable at earlier time points. As stated previously, such chemical-specific gene expression profiles, or ‘chemical fingerprints’, contain mixed molecular events that result from complicated interactions between biological pathways, such as xenobiotic metabolism, stress response, energy metabolism, protein synthesis / degradation, mRNA transcription / degradation, DNA repair / replication and cell growth / cell death control. By comparison with data for prototypical chemicals whose molecular mechanisms of toxicity have been well investigated, one may be able to identify the key gene sets, or TGx biomarkers whose expression levels are highly associated with specific toxicological events, by dissecting the specific molecular pathway from the mixed molecular events. These TGx biomarkers can then be utilized for the evaluation, diagnosis or prediction of toxicity based on their expression changes. For example, carcinogenicity tests in the preclinical stage of drug development require highly time- and labor-consuming tasks, and thus the identification of TGx biomarker genes for carcinogenicity prediction would dramatically reduce R&D time and costs for pharmaceutical companies.

Utilization of TGx Biomarkers

One of the practical applications of TGx biomarkers is to prioritize the drug candidates according to their toxicity profiles based on microarray data. An example is presented in Fig. 2 in which six TGx biomarkers for assessing the
induction of drug metabolizing enzymes, PPARα activation, cell proliferation, glutathione depletion, inflammation or oxidative stress were used to evaluate chemical-induced toxicities in the rat liver. The general trend of the gene expression changes in each biomarker gene set was estimated using the TGP1 score\textsuperscript{174}. The TGP1 score profile for each chemical is visualized by hierarchical clustering in Fig. 2, which demonstrates that each chemical shows characteristic changes in their gene expression levels that are associated with specific toxicity endpoints. Ideally, chemicals showing weaker effects in all the toxicity categories would be promising drug candidates.

In Fig. 3, a model case is presented for identifying a candidate TGx biomarker gene set associated with glutathione depletion, which is known to play a crucial role in acetaminophen (APAP)-type liver injury\textsuperscript{175}. Male F344 rats were treated with the glutathione depletor L-buthionine (S, R)-sulfoximine (BSO), and microarray analysis was conducted on the liver using RG U34A GeneChip. A total of 69 probe sets were identified with signal levels that were inversely correlated with the hepatic glutathione content (Fig. 3A). The validity of the gene set was tested using time-course microarray data for rat livers treated with APAP. As demonstrated in Fig. 3B, 69 probe sets clearly classified the animal groups following APAP treatment and showed that the 24 h APAP group was clustered together with the BSO-treated rats\textsuperscript{113}; this indicates that the gene expression profiles of the APAP-treated (24 h) and BSO-treated rats are very similar and therefore that the 69 gene sets used are associated with glutathione depletion. In another experiment, more detailed TGx data were collected using another the glutathione depleting agent phorone\textsuperscript{114}, and the results of that experiment showed that the ‘glutathione depletion-responsive genes’ maintain a high expression level even after the hepatic glutathione content recovered from acute glutathione depletion immediately after the phorone treatment. Accordingly, it may be better to call these genes ‘glutathione homeostasis-associated genes’ rather than ‘glutathione depletion-responsive genes’ in order to prevent misinterpretation of the microarray results.

Although hierarchical clustering (Fig. 2) and PCA (Fig. 3) are easy to implement, the obtained results are sometimes not conclusive, and the interpretation of the results requires a certain level of proficiency. On the other hand, discriminant analysis, such as SVM, generates conclusive results, such as ‘toxic’ or ‘non-toxic’. The general procedure for SVM analysis is presented in Fig. 4. The first step is to prepare training data sets, such as microarray data for “carcinogenic compounds (positive)” and “non-carcinogenic compounds (negative)”. Next, one develops a ‘classifier’ with these training data sets using the machine learning algorithm of SVM. Once the classifier is developed, a positive / negative outcome can be predicted for a test compound with a known toxicological profile. Although the results produced by a discriminant analysis are conclusive, they are not reliable if the training sets are not selected properly. Furthermore, even when the cross-validation of the established classifier

| Table 3. TGx Biomarkers for Liver Toxicity |
|-------------------------------------------|
| Focused toxicity (tissue or cultured cells) | Species | Reference |
| Gene expression signature | Rat | 19 – 32 |
| Drug metabolizing enzymes | Rat | 33 – 37 |
| Cell injury (multiple mechanisms) | Rat | 38 – 58 |
| | Mouse | 48, 59 – 63 |
| | Human | 39, 64 – 67 |
| Carcinogenicity | Rat | 68 – 82 |
| | Mouse | 83 – 92 |
| | Human | 93, 94 |
| Steatosis / fatty liver | Rat | 95 – 97 |
| | Mouse | 98 – 101 |
| | Human | 102, 103 |
| Oxidative stress | Rat | 104 |
| | Mouse | 105 – 108 |
| | Human | 109 |
| Phospholipidosis | Rat | 110 |
| | Human | 111 – 112 |
| Glutathione depletion | Rat | 113 – 115 |
| | Mouse | 115 |
| | Canine | 115 |
| Fibrosis | Rat | 116 – 122 |
| | Mouse | 123 – 125 |
| | Human | 126 – 132 |
| ER stress | Rat | 133 |
| | Mouse | 134, 135 |
| | Human | 136 |
| Mitochondrial function | Rat | 137 |
| | Mouse | 138 – 140 |
| | Human | 137 |
| PPARα-mediated response | Rat | 76, 141 – 145 |
| | Mouse | 146 – 148 |
| | Canine | 144 |
| Estrogen receptor signaling | Rat | 76, 149 – 151 |
| | Mouse | 152 |
| AhR signalling | Rat | 153 – 157 |
| | Mouse | 156, 158 – 162 |
| | Human | 163 |
| Immune-related response | Rat | 164, 165 |
| | Mouse | 166 – 168 |
| | Canine | 169 |
| Anemia | Rat | 170 |
| Transporters | Rat, Mouse, Human, Monkey, Canine | 171, 172, 173 |

Abbreviations: ER, endoplasmic reticulum; PPAR, peroxisome proliferator-activated receptor; AhR, aryl hydrocarbon receptor.
Fig. 2. Characterization of hepatic toxicity profile. An example of characterizing the hepatic toxicity profile is presented. In this figure, six TGx biomarker gene sets associated with a) phase I drug metabolizing enzyme (DME), b) PPARα-regulated genes, c) cell proliferation, d) glutathione depletion, e) inflammation and f) oxidative stress are used to assess toxicity profiles based on the microarray data for rat livers treated with one of 90 chemicals. The microarray data was retrieved from TG-GATEs, a TGx database developed by the Toxicogenomics Project in Japan (TGP), after obtaining permission. The expression changes for each biomarker set were summarized and estimated using the TGP1 score, and the TGP1 score was subjected to hierarchical clustering. The red and blue colors indicate that the genes included in the TGx biomarker were generally up- or down-regulated, respectively, and the black color indicates that the expression level of the TGx biomarker gene sets did not show characteristic changes as a whole. Ideally, chemicals that do not affect the expression levels of genes included in the TGx biomarker would be desirable drug candidates. This strategy is applied to rank the chemicals based on the toxicity profiling.

Fig. 3. Identification and application of TGx biomarkers for assessing glutathione depletion. A model case for identifying the candidate TGx biomarkers associated with glutathione depletion-type (acetaminophen-type) liver injury is presented. Rats were treated with a glutathione depletor L-buthionine (S, R)-sulfoximine (BSO), and GeneChip analysis was conducted on the liver. (A) A total of 69 probe sets were identified whose signal values were inversely correlated with the hepatic glutathione content. (B) The validity of the 69 probe sets as candidate TGx biomarkers for evaluation of glutathione depletion was evaluated by PCA using time-course microarray data for rat livers treated with acetaminophen. The 69 probe sets clearly classified the animal groups following acetaminophen treatment, and the acetaminophen group was clustered for 24 h together with the BSO-treated rats, suggesting that glutathione homeostasis was highly affected at this time point. Reprinted from Reference, with permission from Elsevier.
certifies good performance for the training data sets used, it may not work if the test compound induces a toxicity whose mechanism is rare or new and has not been considered in the training data sets. For all these reasons, the classifiers should be continuously updated to improve the classification performance.

Microarray Database for TGx Research

To interpret the microarray data appropriately, it is desirable to perform comparative analysis with data obtained from prototypical toxicants. Developing a large-scale reference database, however, is not easy to accomplish, and therefore public databases, such as Gene Expression Ominibus (GEO)\(^1\), ArrayExpress\(^2\), Chemical accomplish, and therefore public databases, such as Gene scale reference database, however, is not easy to obtained from prototypical toxicants. Developing a large-microarray results\(^1\),\(^2\), while others have reported good the inconsistency of interlaboratory / inter-platform reproducibility of microarray datasets across laboratories desirable to perform comparative analysis with data. In addition to public microarray databases, large-scale TGx databases have been developed by collaborative consortiums such as the Toxicogenomics Project in Japan (http://wwwtgp.nibio.go.jp/index.html) and the InnoMed PredTox Consortium (http://www.innomed-predtox.com/), both of which contain microarray datasets for prototypical chemicals as well as proprietary drugs using both in vivo and in vitro systems. Animal and study information as well as microarray data can be retrieved from such databases provided that the TGx datasets were submitted with MIAME-compliant information, a guideline proposed by the Microarray and the Gene Expression Data (MGED) Society\(^1\) to facilitate microarray data sharing. Recently, a number of major scientific journals have begun to require investigators to deposit MIAME-compliant study information as well as microarray datasets at the time of or prior to the submission of manuscripts to their respective journals. This trend will continue because one cannot interpret microarray data appropriately without detailed study information.

Consistency of Microarray Data

Concerns have been raised regarding the reproducibility of microarray datasets across laboratories and microarray platforms. Some papers have reported about the inconsistency of interlaboratory / inter-platform microarray results\(^1\),\(^2\), while others have reported good concordance among laboratories\(^1\),\(^2\) or inconclusive results for this\(^1\),\(^8\). In addition to such laboratory-specific biases, a number of factors cause fluctuations in baseline animal data, such as gender, organ section, strain and fasting state before chemical dosing\(^1\). Furthermore, the vehicle substance used for animal dosing affects the baseline gene expression profile\(^1\), and therefore it is not appropriate to analyze the microarray data sets directly without consideration of the animal study conditions. In this sense, even the MIAME guidelines may not be sufficient for standardizing the TGx study conditions, and additional practical standards may be required to overcome this problem\(^1\).

Even within the same GeneChip platform, the baseline microarray data fluctuates among laboratories. This inconsistency of microarray data is evident among the different generations of rat GeneChips, namely the RG U34A and RAE 230A arrays (Fig. 5A). Practically, we may avoid such inconsistency between two sets of array data by adjusting the median of the signal value between the two datasets (Fig. 5B)\(^1\), and ‘legacy TGx datasets’ can thereby be used together with new datasets.

The MicroArray Quality Control (MAQC) Consortium performed a detailed data comparison in regard to inter / intra-platform microarrays across several laboratories and reported that microarray data shows generally high interlaboratory and inter-platform compatibility if fold-change ranking plus a less stringent statistical cutoff (such as a \(t\)-test) are used to filter the criteria, provided that the expression levels of the filtered genes are relatively high\(^1\). However, other reports have pointed out that the analytical procedure in the MAQC report was inadequate, and therefore the conclusion drawn is questionable\(^1\). In general, however, the reproducibility of interlaboratory microarray data tends to be high when the genes are filtered by fold-change values\(^1\) rather than by stringent \(P\)-values in the statistical analysis.

Species Difference Issues

Because experimental animals are used in preclinical toxicology studies, species differences are always major concerns. A number of papers have reported significant species-specific responses against chemical treatments, even among the rodents. For instance, 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP) acts as a potent phenobarbital-type enzyme inducer in mouse liver but not in the rat or human liver. This species-specific response is associated with the substitution of Thr350 in the mouse constitutive androstane receptor (CAR), a nuclear receptor activated by TCPOBOP, with Met in rat and human CAR\(^1\)–\(^3\). On the other hand, the phenobarbital-type enzyme inducer 2,4,6-triphenyldioxane-1,3 induces hepatic CYP2B in rats but not in mice\(^4\). Since CAR regulates hepatic drug metabolism enzymes and transporters\(^7\), such differential regulation may affect these dramatic species differences in drug metabolism and disposition.

In the case of the estrogenic environmental contaminant \(o,p'\)-DDT, hepatic Cyp17a1 is preferentially upregulated in mice\(^5\) but not in rats\(^6\), even though the majority of orthologous genes exhibit similar gene expression profiles in mice and rats following \(o,p'\)-DDT treatment (Fig. 6). Since CYP17A1 is one of the key steroidogenic enzymes, the mouse-specific upregulation of Cyp17a1 may alter endocrine sex hormone homeostasis. As expected, the blood level of DHEA-S, a precursor of sex hormones produced by CYP17A1, is elevated only in mice\(^7\), and this may lead to endocrine perturbation in addition to the direct estrogenic
Fig. 4. Toxicity prediction by Support Vector Machine algorithm. Support Vector Machine is a popular discriminant analysis algorithm. The first step in this algorithm is to prepare a training data set, such as microarray data for a "carcinogenic compound (positive)" and "non-carcinogenic compound (negative)". Next, a classifier is developed with the training data using the machine learning algorithm. By using the developed classifier, one can predict a positive / negative outcome (carcinogenic / non-carcinogenic outcome in the figure) for a test compound with an unknown toxicological profile. The accuracy of the prediction by the classifier can be estimated by cross-validation using the training data set. Gray and green indicate ‘Positive’ and ‘Negative’ classification areas, respectively. Red spots indicate the support vectors used for the classification of the test data set.

Fig. 5. Overcoming the discrepancy between old and new GeneChip data. Even within the same GeneChip platform, the inconsistency in microarray data is evident among the different generations of rat GeneChips, namely RG U34A and RAE 230A arrays, and this hinders utilization of ‘legacy TGx knowledge’ obtained from older microarrays. (A) The median signal values of the vehicle-treated rats were adjusted between the RG U34A and RAE 230A GeneChip data. The results for 4 representative genes are presented. (B) Principal component analysis using baseline-corrected RG U34A and RAE 230A GeneChip data was performed using the glutathione depletion-associated genes presented in Fig. 3. Adjustment of the baseline signal levels considerably improved the data compatibility between the RG U34A and RAE 230A GeneChip data; the spots for each treated chemical moved closer together (cf. inside area of the dashed circles). Reprinted from Reference189, with permission from Elsevier.
The hepatic CAR mRNA level is decreased in mice but is increased in rats\(^{199}\), and this could result in differential xenobiotic metabolism and disposition in the liver, considering CAR’s role in regulating cassettes of hepatic drug metabolizing enzymes. Thus, marked species differences in hepatic response against chemical treatment have been observed even among rodents, and these phenomena confound the extrapolation of toxicity data from animals to humans. Nevertheless, the identification of potential modes of action as well as species-specific responses may assist in the development or selection of more appropriate models for assessing the toxicity of xenobiotics.

**Future Perspectives**

As the number of TGx biomarkers rapidly increases, some of them will be promising biomarkers that will lead to better understanding of the molecular mechanisms and prediction of toxicity in humans based on preclinical data. However, many of the candidate TGx biomarkers are applicable only to animals, and their feasibility as clinical biomarkers remains unclear. Idiosyncratic drug-induced hepatotoxicity\(^{200}\), which is not detectable in conventional preclinical toxicity studies, is one of the major causes of failure in drug development after the onset of clinical trials, and therefore novel TGx biomarkers which can detect signs of idiosyncratic hepatotoxicity are eagerly awaited.

Recently, seven new renal toxicity biomarkers, including Kim-1, \(\beta\)2-microglobulin and Cystatin C, were officially qualified for particular uses in regulatory decision-making by the US FDA and European Medicines Agency (EMEA)\(^{201}\). These biomarkers were submitted by the Predictive Safety Testing Consortium (PSTC) led by the non-profit Critical Path Institute (C-Path; http://www.c-path.org/). In addition to these novel renal biomarkers, TGx biomarkers for hepatotoxicity will need a similar qualification (or validation) process through collaborative research like that of C-Path.

Identification of TGx biomarkers may lead to the discovery of other biomarkers (genes, proteins or metabolites), the detection of which is easier than measuring hepatic mRNA levels. For example, renal Kim-1 gene expression is upregulated in response to renal injury\(^{202}\), and therefore the Kim-1 mRNA level can be a renal toxicity biomarker. However, Kim-1 protein is also detectable in urine\(^{203}\), and thus the urine Kim-1 protein is a much more convenient biomarker to measure compared with the renal Kim-1 mRNA level. As well, new surrogate hepatotoxicity biomarkers, which are more convenient to detect than hepatic mRNA, could be discovered through a profound understanding of the molecular mechanisms of toxicity by utilizing TGx mRNA biomarkers. ‘Ideal’ TGx biomarkers for hepatotoxicity will be those that are sensitive, specific, predictive and, above all, ‘extrapolatable’ to humans, and it is the responsibility of pharmaceutical toxicologists to discover/establish novel biomarkers to assist in the improvement of risk assessment in humans.
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