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Review article

DNA microarray analysis as a tool to investigate the therapeutic mechanisms and drug development of Chinese medicinal herbs

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ABSTRACT

Chinese herbal medicines have been used for the treatment of various diseases for centuries. Although several herbal formulas and herbal components have shown therapeutic potential, the active components and the molecular mechanisms mediating the effects of said formulas remain to be discovered. Microarray analysis has become a widely used tool for the generation of gene expression data on a genome-wide scale. This paper discusses the application of whole genome expression profiling as a tool to investigate the molecular mechanisms governing the therapeutic effects of traditional Chinese medicine. This review also highlights how data derived from DNA microarray analysis can be used to screen for drug targets of various herbal drugs, to predict the therapeutic potential of herbal drugs, to analyze the safety of drugs in the preclinical stage of drug development, and to establish a modern definition of traditional Chinese medicine.

1. Introduction

Systems biology serves as a translational platform between traditional Chinese medicine and modern science. In this study, we review the technology behind whole genome expression profiling and discuss the biomedical application of the technique to the study of Chinese medicinal herbs.

2. Technology behind genome expression profiling

2.1. *Development of whole genome expression profiling*

In 1995, Schena and colleagues[1] at Stanford University in Palo Alto, CA, USA, published the first paper on the use of

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cDNA microarray probes printed in a two-dimensional grid onto glass slides. They showed that their high-capacity system could simultaneously monitor the expression of many genes. Microarrays prepared by high-speed robotic printing of complementary DNAs on glass are used for measurements of quantitative expression of corresponding genes. Because of the small format and the high density of arrays, hybridization volumes of less than two microliters can be used, enabling the detection of rare transcripts in probe mixtures derived from two micrograms of total cellular mRNA. Two-color fluorescence hybridization is then used to simultaneously visualize differentially expressed genes. In 1996, Affymetrix began to market commercially available DNA chips. Various microarray experimental platforms have been developed since then.

2.2. Commonly used microarray platforms

Three different types of microarray platforms are commonly used: spotted cDNAs, spotted oligonucleotides, and Affymetrix arrays [2].

Spotted cDNA arrays typically use sets of specific cDNA plasmids in gridded liquid. The inserts of each clone are typically amplified by polymerase chain reaction, and a few pico liters are physically spotted onto glass slides by liquid-handling robots. Spotted cDNA arrays are only used in academic centers because of their flexibility and relatively low cost.

Spotted oligonucleotide arrays are also built on glass slides by liquid-handling robots; however, the input solution comprises synthetic oligonucleotide (often 60–70 mer) rather than plasmids. Most of the process is automated, leading to less sample mix-up and less sample dropout. Disadvantages of spotted oligonucleotides include the relatively high cost of synthesizing large numbers of large oligonucleotides and the nonrenewable nature of the resource. Nonetheless, spotted oligonucleotide arrays are still widely used.

Affymetrix GeneChips are factory designed and synthesized. Design is done using software to choose a series of 11- to 25-mer probes from the 3-foot end of each transcript or predicted transcript in the genome. Synthesis of arrays is done using light-activated chemistry and photolithography methods. Spotted oligonucleotides and Affymetrix arrays have superseded the use of spotted cDNAs. The manufacturers of commonly used DNA microarray platforms are listed in Table 1.

2.3. Limitations and standardization of microarray platforms

DNA microarrays enable researchers to simultaneously monitor the expression of thousands of genes. However, the current technology has several limitations. The major problems are sensitivity, accuracy, specificity, and reproducibility of microarray results. Studies have shown that, for relatively abundant transcripts, the existence and direction, but not the magnitude, of expression changes can be reliably detected. However, accurate measurements of absolute expression levels and the reliable detection of low abundance genes are difficult to achieve. The main problems seem to be the suboptimal design or choice of probes and some incorrect probe annotations. Marshall [3] compared the reliability of numerous array platforms, including the Affymetrix GeneChip, the Agilent array and the Amersham array systems, and found that more than one-half of the variability observed in the results was attributable to differences in the microarray platforms themselves. Efforts to standardize microarray data have been underway for some time and include the standardization of sample preparation, RNA isolation, cDNA synthesis, hybridization analysis, and quality control checkpoints to ensure reproducibility of data. For example, quality control criteria for RNA isolation include yield, purity, and integrity. An RNA integrity number greater than eight indicates that the RNA sample is suitable for cDNA synthesis. The criteria for cDNA labeling include concentration and incorporation efficiency. An incorporation efficiency of 15 labeled nucleotides per 1000 cDNA nucleotides indicates that cDNA labeling is suitable for hybridization. The gene expression profile obtained using standardized protocols can yield data that are consistent between laboratories and are intrinsically comparable [4].

Use of identical microarray chips and identical protocols would minimize the efforts made by researchers to integrate expression data, thereby allowing for the information embedded in these data to be maximally explored. In 2004, the Microarray and Gene Expression Data (MGED) society wrote an open letter to scientific journals proposing standards for publication. The MGED society suggested that journals require

Table 1 – Manufacturers of DNA microarray platforms.

Manufacturer	Location	Website
Affymetrix	Santa Clara, CA, USA	www.affymetrix.com
Agilent Technologies	Santa Clara, CA, USA	www.agilent.com
Expression Analysis	Durham, NC, USA	www.expressionanalysis.com
Jivan Biologics	Larkspur, CA, USA	www.jivanbio.com
Marligen Biosciences	Ijamsville, MD, USA	www.marligen.com
NanoString Technologies	Seattle, WA, USA	www.nanostring.com
NimbleGen	Madison, WI, USA	www.nimblegen.com
Oxford Gene Technology	Oxford, UK	www.ogt.uk
PerkinElmer	Waltham, MA, USA	www.perkinelmer.com
Phalanx Biotech Group	Hsinchu, Taiwan	www.phalanxbiotech.com

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submission of microarray data to one of two public repositories: Gene Expression Omnibus (GEO) or ArrayExpress. Moreover, they stated that authors should provide a checklist of variables and supply the checklist as supplementary information at the time of submission. Other members of the microarray community welcomed these steps, in particular Brazma and colleagues [5], who proposed the Minimum Information About a Microarray Experiment (MIAME), a guideline that describes the minimum information required to ensure that microarray data can be easily interpreted. The standardization of global gene expression data will make microarray data much more useful and accessible.

In summary, DNA microarray technology has evolved rapidly since its introduction in 1995. Although certain limitations of the current technology exist and have become more apparent during the past couple of years, the ability of microarrays to monitor the expression of thousands of genes simultaneously is unsurpassed [6].

3. Application of whole genome expression profiling to traditional Chinese medicine studies

Whole genome expression profiling can be applied to study the biomedical effects of Chinese medicinal herbs. Extracts prepared from medicinal plants and other natural sources contain a variety of molecules with potent biological activities. Unfortunately, it is often difficult to analyze the biologic activities of these extracts because of their complex nature and the possible interaction of their components. Genome-wide expression monitoring with high-density microarrays provides a simple way to test the biochemical effects of herbs, thereby gaining insight into their potential beneficial effects and negative side effects. DNA microarray has been used to evaluate the toxicity of novel drug candidates and to identify disease targets for drug development. Additionally, the therapeutic efficacy of a given drug can be predicted on the basis of gene expression patterns *in vitro*.

3.1. *Evaluation of biologic activity and mechanisms of Chinese herbs*

Microarray data have been used to characterize the biologic activities and mechanisms of action of herbal formulae or herbal compounds. For example, PC-SPES is a dietary supplement comprised of extracts from eight different herbs: *Scutellaria baicalensis*, *Glycyrrhiza glabra*, *Ganoderma lucidum*, *Isatis indigotica*, *Panax* pseudo-ginseng, *Dendranthema morifolium*, *Rabdosia rebescens*, and *Serenoa repens*. PC-SPES is also used as an alternative therapy by patients with prostate carcinoma [7–9]. The gene expression profile in cultured cells that have been exposed to PC-SPES shows differential expression of genes involved in modulating cell cycle, cell structure, and androgen response, indicating that alteration of some of those genes may be responsible for PC-SPES-mediated cytotoxicity [10]. Yukmijihwang-tang (YMJ), also known as LiuWei Dihuang Wang, is composed of six different medicinal herbs, including *Rehmannis radix*, *Radix dioscoreae*, *Fructus corni*, *Poria*, *Cortex moutan*, and *Radix alismatis*. YMJ has been widely used for centuries as an antiaging herbal formula in Asian countries [11]. Microarray data indicate that YMJ enhances memory retention by inducing several genes that are involved in protecting neuronal cells, enhancing cell proliferation, and stimulating neurite growth [12]. *Pinelliae Rhizoma* extract (PRe) is used to treat cough and asthma. However, the mechanism by which PRe exerts its effect on psychological disorders has not been studied. Kim and coworkers [13] used microarray to analyze the effect of PRe in mice exposed to psychological stress. They found that the expression of most genes that are altered in response to psychological stress is restored to normal levels in PRe-treated mice, with recovery rate of 81.5% for up-regulated genes and 85.2% for down-regulated genes. When the interaction network was analyzed, the recovery rate of the core node genes (46 up- and 29 down-regulated genes) in PRe-treated mice was over 95%, indicating that those genes may be the effective targets of PRe. Curcumin, a major chemical component of *Curcuma longa*, is used as a spice to give a specific flavor and yellow color to curry. It is also used as a cosmetic agent and in some medical preparations [14]. Curcumin displays anticarcinogenic properties in animals [15,16]. Microarray-based gene expression patterns indicate that, in addition to anticarcinogenic effects, curcumin may be an effective anti-metastatic agent via the regulation of expression of certain genes [17]. Aristolochic acid (AA), the major constituent of *Aristolochia* species, is associated with nephritis and renal cancer [18–20]. Microarray and network analysis have shown that most AA-altered genes are connected with nuclear factor-κB (NF-κB), suggesting that NF-κB plays a critical role in the pathogenesis of AA-induced renal diseases [21]. Extracts prepared from medicinal plants and other natural sources contain a variety of molecules with potent biological activities; the aforementioned studies suggest that genome-wide expression monitoring with high-density microarrays is an effective method for analyzing the biologic activities of those extracts.

3.2. *Establishing a modern definition of traditional Chinese medicine*

Chinese herbal formulas consist of several herbal components. However, the mechanisms of action of most Chinese herbal formulas and the relationship between formulae and their components remain to be elucidated. The putative mechanism of San-Huang-Xie-Xin-Tang (SHXXT) and the relationship between SHXXT and its herbal components were analyzed in our laboratory using a microarray technique [22]. Gene-set enrichment analysis indicated that SHXXT and its components displayed a unique anti-proliferation pattern involving p53 and DNA damage signaling pathways in HepG2 cells. Network analysis showed that SHXXT-affected genes were regulated by p53. In addition, clustering analysis showed that *Rhizoma coptis*, the principal herb in SHXXT, shared a similar gene expression profile with SHXXT. These findings indicate that *R coptis* is the principal herb in the herbal combination SHXXT (Fig. 1). To the best of our knowledge, this was the first study to reveal the relationship between a traditional Chinese medicine formula and its herbal components using microarray and bioinformatics approaches.

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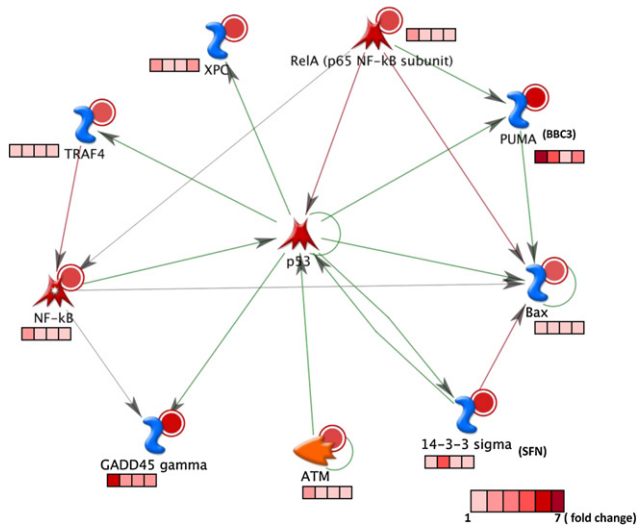

Fig. 1 – Network analysis of SHXXT-regulated genes. We selected the target genes that are regulated by p53 from http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchTFGeneForm. To estimate the overall regulatory effect of SHXXT on these target genes, we used the 'geneSetTest' function implemented in the R program of the Limma package to compare the absolute t-statistic values for these target genes with those for all genes. These target genes were then combined with the differentially expressed genes, which belonged to the Gene Ontology (GO) category 'regulation of biological process,' to investigate their relationship with p53. We used the MetaCore Analytical suite to construct the interaction networks between p53-downstream genes and part of the differentially expressed genes. The fold changes in gene expression in SHXXT-, *Rheum officinale*-, *Coptis chinensis*-, and *Radix scutellariae*-treated cells, respectively, are shown at the bottom. SHXXT = San-Huang-Xie-Xin-Tang.

3.3. *Evaluation of drug safety*

Many natural products, including polyphenols, terpenes, alkaloids, flavonoids, and phenolics, are potential therapeutic agents [23]. Previous studies have shown that phytochemicals affect the expression levels of genes involved in drug metabolism [24]. To evaluate whether phytochemicals affect drug metabolism, we analyzed the expression levels of genes encoding phase I and II drug metabolism enzymes in cells exposed to anthraquinone compounds. Phase I drug metabolism genes encode alcohol dehydrogenases, aldehyde dehydrogenases, and cytochrome P450 families, while phase II drug metabolism genes encode glutathione S-transferases, sulfotransferase, and UDP glucuronosyltransferase (UGT) families. We found that genes involved in phase II drug metabolism were down regulated during anthraquinone compound treatment (Table 2). These data suggest that anthraquinone compounds may slow down the excretion of drugs, thereby increasing the half-life of drugs [25].

Table 2 – Analysis of expression levels of genes associated with drug metabolism.[a]

Gene symbol	$\log_2$ ratio	Standard deviation
UGT1A10	−0.23	1.89
UGT2A1	−0.28	1.15
UGT2B11	−1.70	5.42
UGT2B15	−0.85	0.69
UGT2B4	−0.34	0.29
UGT2B7	−0.94	0.79

a Results were obtained from three independent assays. A total of 219 genes associated with drug metabolism were selected from 'The Pharmacogenetics and Pharmacogenomics Knowledge Base' website (https://www.pharmgkb.org/index.jsp). Among these genes, we analyzed the expression levels of phase I drug metabolism genes, including alcohol dehydrogenases, aldehyde dehydrogenases, and cytochrome P450 genes, and phase II drug metabolism genes, including glutathione S-transferases, sulfotransferase, and *UGT* genes. The $\log_2$ ratio and standard deviation of *UGT* genes are shown.

3.4. *Prediction of the therapeutic potential of medicinal herbs*

Vanillin has been shown to inhibit mutagenesis and to suppress the invasion and migration of cancer cells [26]. In our previous studies, microarray data and gene ontology investigation indicated that vanillin affected clusters of genes involved in the cell cycle and apoptosis. Network analysis indicated that Fos might play a central role in the regulation of the gene expression network. Results from reporter assay and Western blot further indicated that vanillin inhibited Fos-related transcription factor activator protein 1 (AP-1) activity via an extracellular signal-regulated kinase pathway. Our data suggest that vanillin exhibits anticancer potential by regulating cell cycle and apoptosis and that its regulation may involve the suppression of AP-1 (Fig. 2) [27,28].

AA belongs to a family of compounds found in the *Aristolochiaceae* family of plants. *Aristolochia* species in particular have been used for centuries in Asia for medicinal purposes. Although AA is bioactivated in both the kidney and liver, it only induces diseases and tumors in kidney and urinary tract in human and rodents [18]. To elucidate why AA displays such tissue-specific carcinogenicity, Chen and colleagues [29] examined gene expression profiles in kidney and liver of rats treated with carcinogenic doses of AA. They found that the biologic processes related to defense response, apoptosis, and immune responses were significantly altered by AA exposure in kidney but not in liver. These findings may explain why AA induces tumors in the kidney but not in the liver [29].

Ginkgo biloba extract EGb 761 is wildly used to treat neurologic disorders [30,31]. In a previous study, we tested the effects of EGb761 on the transcriptional profile of mouse genes. A KEGG pathway analysis showed that EGb761 affected the neuroactive ligand-receptor interaction pathway in brain. A total of 53 genes were significantly affected, and EGb761 up-regulated a subgroup of dopamine receptors, especially dopamine receptor 1a. Immunohistochemical staining confirmed the microarray data. The finding that *G biloba*

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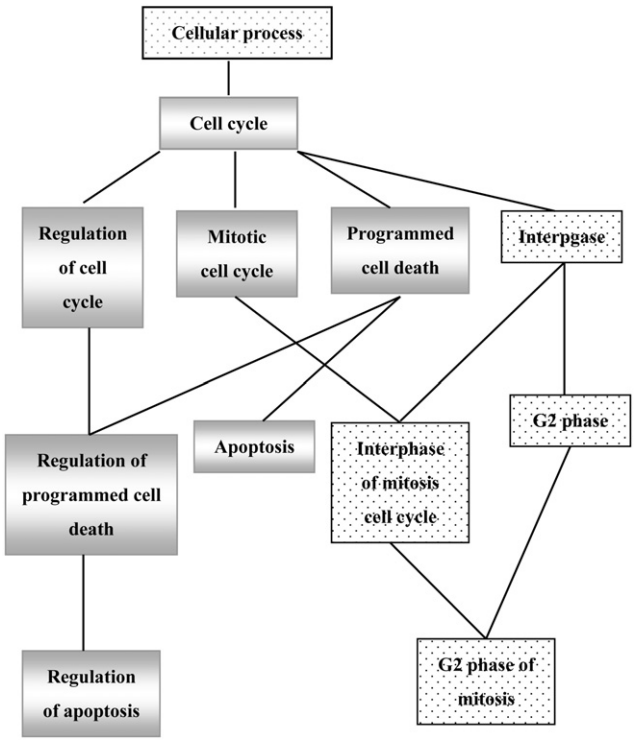

Fig. 2 – Ontology analysis of vanillin-affected genes. vanillin-affected genes were analyzed by GO on the Gene Ontology Tree Machine website (http://bioinfo.vanderbilt.edu/gotm/), a web-based and tree-based data-mining environment for gene sets. We used the WebGestalt tool to test significant GO terms, and the significant GO terms are shown.

treatment resulted in increased expression of dopamine receptor 1 in brain may explain why EGb761 is an effective treatment of neurologic disorders such as Parkinson disease (Table 3) [32].

3.5. *New drug development*

Whole genome expression profiling has also been used for the development of new drug [33–36]. Large-scale gene expression analyses of toxin-treated cells and animals have yielded information on the toxic potential of novel drug candidates [37–41]. In addition, gene expression profiles have been applied to identify the disease targets for drug development [42]. Moreover, the therapeutic efficacy of drugs can be predicted on the basis of gene expression signatures *in vitro* [43,44].

A number of studies have shown that DNA microarray data have potential utility in drug discovery and drug target validation [44,45]. For example, Lamb and others [46] analyzed the expression profiles of 164 small molecules with DNA microarray. By comparing the genomic signatures of drug candidates or the disease state to this resource, the authors found that it was possible to identify potential mechanisms of action, confirm previous applications of known drugs, and identify additional potential uses for known drugs [46]. Their results demonstrate that the establishment of a huge gene expression database would be useful for finding connections among small molecules that share similar mechanisms of action and that are involved in similar physiologic processes, thereby allowing for the development of disease-fighting drugs.

Several studies have indicated similarities between gene expression profiles and therapeutic activities [46–48]. In addition, genome-wide expression monitoring with high-density microarrays provides a simple way to test biochemical effects of herbs, thereby gaining insights into their potential beneficial effects and negative adverse events [30]. In a recent study, we applied DNA microarray to analyze biologic events, predict the therapeutic potential of drugs, and evaluate the safety of herbal formulas [49]. For seven consecutive days, mice were administrated orally with 15 of the most widely used Chinese herbal formulae listed in the Taiwan National Health Insurance Database, and the gene expression profiles in liver or kidney were analyzed by DNA microarray. Our data showed that most formulas altered metabolic pathways, such as the pathways governing glutathione metabolism and oxidative phosphorylation, and regulatory pathways, such as that regulate antigen processing and presentation and insulin-like growth factor signaling. By comparing the gene expression signatures of formulas with those of disease states or drugs, we found that response of mice to formula might be associated with disease state in said mice, such as metabolic or cardiovascular diseases. Moreover, most formulas altered the expression levels of cytochrome P450, glutathione S-transferase, and *UGT* genes, suggesting that caution should be paid to possible drug interactions of these formulas. Furthermore, the similarities in gene expression profiles between formulas and toxic chemicals were low in kidney, suggesting

Table 3 – Neuroactive ligand-receptor interaction of EGb761-affected genes in the brain and kidney.[a]

	Observed (total)	*p* value
Brain	53 (237)	4.13×10^{6}
Kidney	0 (237)	0.536

a Fluorescent RNA targets were prepared from 5 μg of total RNA using a MessageAMP aRNA kit and Cy5 dye. Fluorescent targets were hybridized to Mouse OneArray Whole Genome DNA microarray. After an overnight hybridization at 50 °C, nonspecific binding targets were washed away and the slides were scanned with an Axon 4000 scanner. The Cy5 fluorescent intensity of each spot was analyzed by genepix 4.1 software. The signal intensity of each spot was corrected by subtracting background signals. Spots with a signal-to-noise ratio of less than 0 as well as those of control probes were filtered. Spots that passed these criteria were normalized by the R program in the Limma package using quantile normalization [50]. The *p* value of each gene was calculated by *t*-statistics using the Differential Expression (T-Rex) tool in the Gene Expression Pattern Analysis Suite [51]. These differentially expressed genes ($p < 0.01$) were further analyzed by the KEGG pathway [52]. Pathway enrichment analysis was performed on the WebGestalt website (http://bioinfo.vanderbilt.edu/webgestalt/login.php) by the hypergeometric test, which is used to evaluate the *p* value of the over-represented pathways. The neuroactive ligand-receptor interaction pathway is shown.

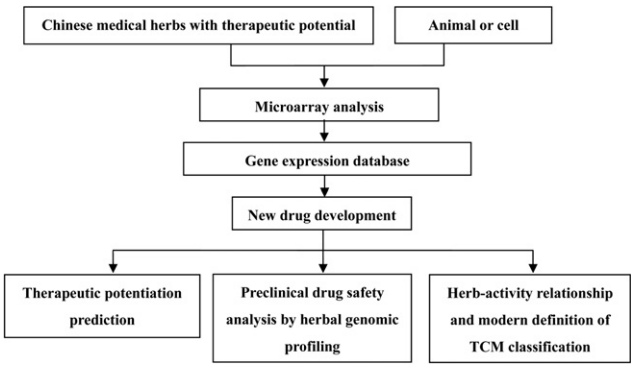

Fig. 3 – Paradigm for the application of whole genome expression profiling as a tool for therapeutic prediction, drug development, and safety evaluation of Chinese herbal medicines.

that these formulas might not induce nephrotoxicity in mice. This transcriptomic platform will not only help researchers understand the therapeutic mechanisms associated with herbal formulas and gene interactions, but will also help researchers develop novel disease-fighting drugs (Fig. 3).

4. Conclusion

Whole genome expression profiling can provide a basis for investigating the molecular mechanisms governing the therapeutic effects of Chinese herbal medicines and can be used to elucidate the biology of disease progression, identify potential therapeutic targets, and facilitate the development of traditional Chinese medicine–derived biopharmaceutical products.

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