Review

Microarray analysis of gene expression in medicinal plant research

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ABSTRACT: Expression profiling analysis offers great opportunities for the identification of novel molecular targets, drug discovery, development, and validation. The beauty of microarray analysis of gene expression is that it can be used to screen the expression of tens of thousands of genes in parallel and to identify appropriate molecular targets for therapeutic intervention. Toward identifying novel therapeutic options, natural products, notably from medicinal plants used in traditional Chinese medicine (TCM), have been thoroughly investigated. Increased knowledge of the molecular mechanisms of TCM-derived drugs could be achieved through application of modern molecular technologies including transcript profiling. In the present review, we introduce a brief introduction to the field of microarray technology and disclose its role in target identification and validation. Moreover, we provide examples for applications regarding molecular target discovery in medicinal plants derived TCM. This could be an attractive strategy for the development of novel and improved therapeutics.

Keywords: Microarray, traditional Chinese medicine

1. Introduction

The 30,000-40,000 genes of the human genome project constitute the likely possible therapeutic targets for medicine (*1,2*). The continuous gain of information on the sequence of entire genomes has increasingly challenged researchers to identify the functions of these genes and their interaction pathways in health and disease (*3*). Ways to measure gene expression include: northern blotting, serial analysis of gene expression (SAGE) differential display, and dot-blot analysis. All previously mentioned techniques are inappropriate for the analysis of the expression of multiple genes at once. The emergence of new tools enables investigators to address previously intractable problems and to reveal novel potential targets for therapies. Nowadays, microarray technology can be used to test thousands of genes at the same time and to analyze the expression of those genes. Microscopic arrays of large sets of DNA sequences immobilized on solid substrates are becoming a standard technology applied in research laboratories all over the world. Since its first application (*4*), microarray technologies have been productively functional in almost each and every aspect of biomedical research (*5-9*). Arrays are ordered samples of DNA sequences with each sample representing a particular gene. These arrays can then be assayed for changes in gene expression of the representative genes after various treatments, various conditions or tissue origins, thus providing a functional aspect from sequence information in a given sample (*10*).

DNA microarrays are precious tools in the identification or quantification of many specific DNA sequences in complex nucleic acid samples (*11*) therefore, they have been used to identify cardinal aspects of growth and development, as well as to explore the underlying genetic causes of many human diseases (*5*). Microarray based studies have enormous potential in the exploration of disease processes such as cancer (*6*) and in drug design, response, and development (*7*). In addition, the technology is applied to a considerable extent to investigate several pathological conditions, such as inflammation (*12*), breast cancer (*8*), colon cancer (*13*), and pulmonary fibrosis (*14*). Microarrays, comprising thousands of genes at once, generate gene expression 'profiles'. Such profiles are comprehensive patterns that are characteristic of the responses of cells or tissues to drug treatment, to environmental changes, to differentiation into specialized tissues, or to dedifferentiation into tumor cells. Thus, microarrays document detailed responses of cells and tissues to both disease and the intended and unintended effects of drug treatments and hence facilitate medical research (*3,7,15*). Moreover, such trials expand the size of existing gene families, discover new patterns of coordinated gene expression across gene families, and disclose entirely new classes of genes.

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2. Microarray design

Most arrays used for gene expression profiling and molecular targets analysis in the biological sciences today can be divided into two groups: complementary DNA (cDNA) and oligonucleotide microarrays (*16*). This division refers to characteristics of the probes, the individual pieces of gene-specific DNA that are spotted on the array surface. cDNA probes are usually products of the polymerase chain reaction (PCR) generated from cDNA libraries or genomic DNA, and are typically more than 150 nucleotides in length. On the other hand, synthetic oligonucleotides have a maximum length of around 80 nucleotides, thus conferring greater specificity among members of gene families (*17,18*). Array fabrication involves either spotting of presynthesized probes using highly precise robotic spotters, or in situ synthesis on glass slides (*16*). High-density spotted cDNA microarrays can contain up to 40,000 probes on a conventional microscope slide. In contrast, oligonucleotide arrays, consisting of gene-specific oligonucleotides, are synthesized directly onto a solid surface by either photolithography or ink-jet technology (*19*). Probes can be designed that represent the most unique part of a given transcript, making the detection of closely related genes possible (*16*). A major advantage of oligonucleotide arrays over cDNA arrays is that they require no handling and tracking of cDNA resources (*19*). Furthermore, the use of synthetic reagents in the manufacturing of oligonucleotide arrays minimizes variation among arrays, thus ensuring a high degree of reproducibility between microarray experiments.

Sample preparation is similar for cDNA and oligonucleotide microarrays. In both cases, mRNA is extracted, purified, reverse transcribed to cDNA, labeled, and hybridized to probes on the surface of the array slide (*16*). Two fluorescent dyes allow cDNA from two treatment populations to be labeled with different colors (Figure 1). When mixed and hybridized to the same array, the differentially labeled cDNA results in competitive binding of the target to the probes on the array. After hybridization, the slide is imaged using a confocal laser scanner and fluorescence
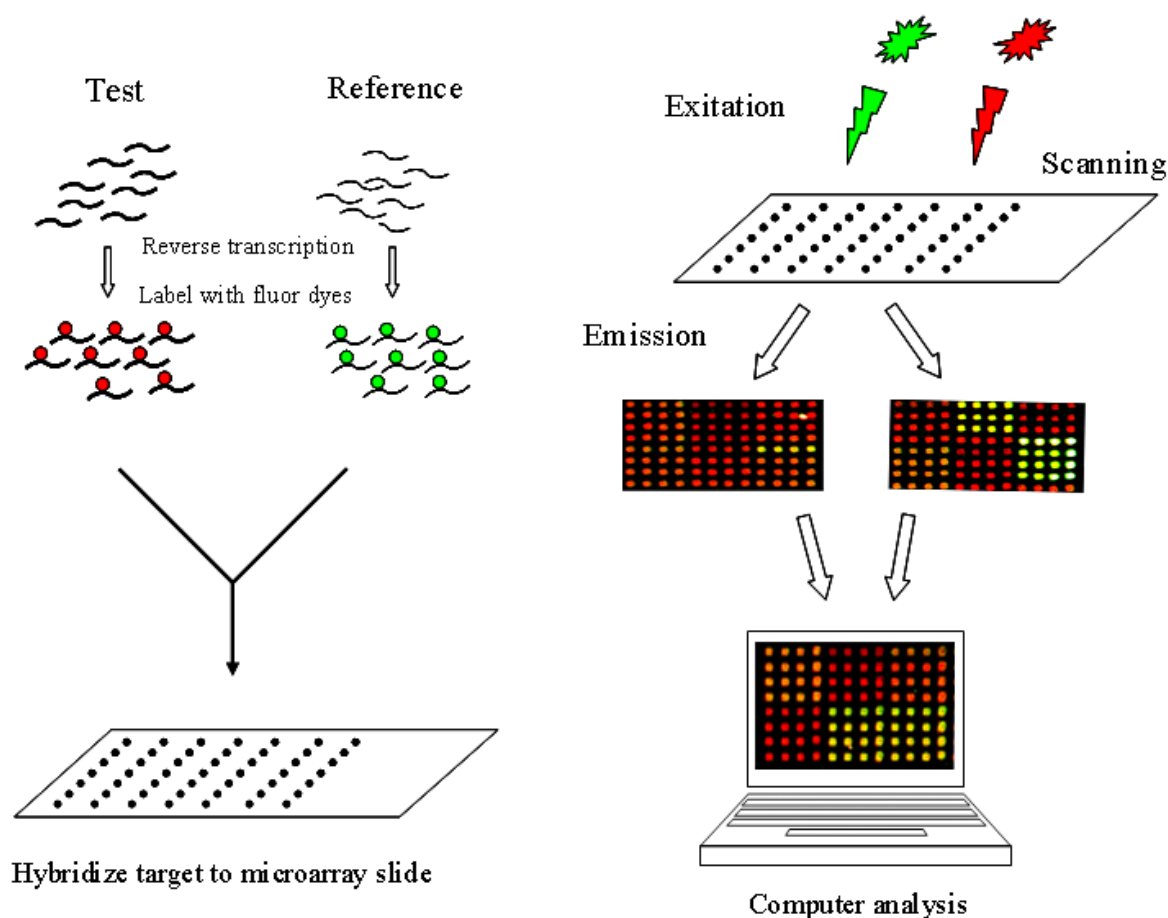

Figure 1. cDNA microarray procedure. Templates for genes of interest are amplified by PCR then printed on coated glass microscope slides. Total RNA from both the test and reference sample is fluorescently labeled with different fluor dyes. The fluorescent targets are pooled and allowed to hybridize to the clones on the array slide. Laser excitation of the incorporated targets yields an emission with a characteristic spectrum, which is measured using a scanning confocal laser microscope. Monochrome images are imported into software in which the images are merged. Data from a single hybridization experiment is viewed as a normalized ratio. In addition, data from multiple experiments can be examined using any number of data mining tools.

measurements are made separately for each dye at each spot on the array (Figure 1). This dual labeling enables the ratio of transcript levels for each gene on the array to be determined (*16,20*). Specialized software and data management tools are then used for data extraction, normalization, filtering, and analysis (*21*).

3. Identification of potential therapeutic targets

Recently, the search for disclosing therapeutic targets and discovering new therapeutic options has relied mainly on approaches based on large-scale genomics including the sequencing of expressed-sequence tags (ESTs), serial analysis of gene expression (SAGE) differential display, homology cloning, and related approaches (*22*). These EST sequencing efforts resulted in availability of databases containing information on the majority of human genes (*23*), most of which were of unknown therapeutic significance. Although these bioinformatics approaches, based on sequence homology and structural motifs, are conveniently and extraordinarily valuable, investigators still need an experimental approach to prioritize potential therapeutic targets. Microarray technology provides an excellent solution because it facilitates the identification of novel potential therapeutic targets from tens of thousands of genes in a single experiment. Using microarray technology to screen for differentially expressed genes is widely acknowledged as a valuable approach in the target discovery process (Table 1).

Forward pharmacology is an approach that can assist in identifying the mechanisms of action of poorly understood drugs or other bioactive compounds. It involves using microarrays to monitor mRNA changes induced by drugs or other bioactive compounds in order to deduce previously unknown actions of these compounds (*7*). For example, Hughes *et al.* (*24*) assessed the levels of more than 6,000 transcripts including 279 gene knockout strains under 300 experimental conditions. The global expression profiles of these diverse experimental conditions were then used for monitoring the effects of several drugs on yeast where dyclonine, a topical anesthetic of unknown action, was found to be implicated in perturbation of the pathway of ergosterol metabolism. This hypothesis was further confirmed by similar observations of the effects of haloperidol on the same pathway (*24*).

4. Microarray analysis after therapeutic target validation

Several approaches can be taken to validate and to prioritize candidate therapeutic targets once a shortlist has been identified. Among the most important approaches to validate and to prioritize candidate therapeutic targets are gene knockout and knock-in strategies in cells, model organisms and mice. In this context, microarrays can be used to assess the primary and secondary consequences of genetic manipulation.

5. Microarray in TCM

TCM has been used for thousands of years in China and is currently widely practiced in Chinese cancer centers. Nevertheless, it is a brand new area for formal scientific evaluation. TCM represents a holistic approach and lacks high-quality scientific evidence for its effectiveness. Therefore, TCM is frequently regarded with some skepticism by western academic medicine (*25*). Since DNA chip technologies has been introduced into medical sciences (*4,26*), many researchers have applied this technology to pharmacological analyses (*10,12*). Recent studies tried to cross the bridge between TCM and modern western medicine through applying modern technologies to identify molecular mechanisms and novel targets involved in TCM action on different

Table 1. Types of microarray and examples of current applications

Microarray Type	Sample	Objective	Applications
Expression profiling (cDNA, oligonucleotide arrays)	mRNA	To determine the changes in gene expression between different disease or different treatment conditions	Tumor classification Identification of prognostic and predictive markers Drug responce
SNP analysis	Genomic DNA	To detect mutations or polymorphisms in a gene sequence	Determination of genetic predisposition to a disease Monitoring of disease progression Drug development
Resequencing arrays	Genomic DNA	To sequence portions of the genome	Evaluation of germline mutations in individuals Identification of somatic mutations in cancer
Array CGH	Genomic DNA	To identify genetic amplifications, deletions or copy number changes	Tumor classification Risk assessment Development of prognostic and predictive markers
Protein microarrays	Antibody	To determine the changes in protein expression between different disease or different treatment conditions	Tumor classification Identification of prognostic and predictive markers Drug development

Abbreviations: CGH, comparative genomic hybridization; SNP, single nucleotide polymorphisms.

disorders. Some of these studies are presented here to pinpoint the significance of applying high throughput technologies in medicinal plant research.

6. Berberine and microarray

Berberine, a natural isoquinoline alkaloid, has been found in many clinically important plants including *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), *Coptis chinensi*s (goldenthread or Coptis), and *Coscinium fenestratum* (*27,28*). Berberine exhibits a wide range of biochemical and pharmacological activities (*29,30*). Berberine has been reported to be used as an anti-arrhythmia, anti-hypertension, anti-diarrhea, and anti-inflammatory agent (*31-33*). Additionally, the natural product was reported to possess an anti-tumor activity against different tumor types (*34-36*). To better understand the physiological actions and the molecular targets of berberine in pancreatic cancer, Iizuka *et al.* (*37*) used an oligonucleotide array which contained approximately 11,000 genes and undertook a gene expression profiling study to monitor the expression changes associated with sensitivities to berberine and *Coptidis rhizoma* in 8 human pancreatic cancer cell lines. From the oligonucleotide array data, 20 and 13 genes with strong correlations ($r^2 > 0.81$) to ID_{50} values for berberine and *C. rhizoma* were selected, respectively. Among these 33 genes, the levels of expression of 12 were correlated with the ID_{50} values of both berberine and *C. rhizoma*. They concluded that these genes are associated with the tumor-killing activity of berberine in *C. rhizoma* (*37*). Moreover, expression of the remaining 21 genes was correlated with the ID_{50} value of either purified berberine or *C. rhizoma*. Such an approach allowed common and distinct genes responsible for anti-proliferative activities of purified berberine and *C. rhizoma* to be identified (*37*).

Recently, DNA microarray chips were utilized to investigate the transcriptional changes of *Y. pestis*, a gram negative coccobacillar bacterium that causes plague, in response to berberine. The analysis was done after exposing *Y. pestis* to berberine. A total of 360 genes were differentially expressed; 333 genes were up-regulated, and 27 were down-regulated. The up-regulation of genes that encode proteins involved in metabolism was a remarkable change. Genes encoding cellular envelope and transport/binding functions represented the majority of the altered genes in addition to a number of genes of unknown encoding or unassigned functions. Furthermore, a number of genes related to iron uptake were also induced (*38*).

7. PC-SPES and microarray

PC-SPES is a preparation of eight Chinese herbs used in the treatment of prostate cancer and exhibits a promising antiproliferative and antitumor activity *in vivo* and *in vitro* in diverse cancer types (*39*). Applying DNA chip technology, Bonham *et al.* investigated the molecular effects of the herbal compound PC-SPES on prostate carcinoma cells. cDNA microarray analysis was utilized to identify expression profiling changes in LNCaP prostate carcinoma cells treated with PC-SPES and estrogenic agents including diethylstilbestrol. Interestingly, PC-SPES altered the expression of 156 genes following 24 h of exposure. Of particular interest, transcripts encoding cell cycle-regulatory proteins, alpha- and beta-tubulins, and the androgen receptor were significantly down-regulated. After comparing the gene expression profiles patterns resulting from these treatments, they concluded that the herbal preparation PC-SPES exhibits activities distinct from those of diethylstilbestrol and suggested that alterations in specific genes involved in modulating cell structure, cell cycle, and androgen response could be responsible for PC-SPES-mediated cytotoxicity (*40*).

8. Curcumin and microarray

Curcumin (diferuloylmethane), a major chemical component of turmeric (*Curcuma longa*), is used as a spice to give a specific flavor and yellow color to curry. It has also been used as a cosmetic and in some medical preparations (*41*). Curcumin has been shown to display anticarcinogenic properties in a mouse model system, as indicated by its ability to inhibit phorbol ester-induced skin tumors (*42*). In addition to its anticarcinogenic effect, curcumin exhibits antioxidant, antiangiogenic, antiproliferative, and anti-inflammatory properties (*43*). The previously mentioned effects of curcumin may be mediated by its inhibitory effects on a host of cell-signaling factors, including c-Myc, transcription factor AP-1, NF-κB, protein kinase C, Egr-1, epidermal growth factor receptor tyrosine kinase, c-Jun *N*-terminal kinase, protein serine/threonine kinases, protein tyrosine kinases, and IκB kinase (*42,44,45*).

A microarray analysis of gene expression profiles were used to characterize the anti-invasive mechanisms of curcumin in the highly invasive lung adenocarcinoma cells (CL1-5) (*46*). Using microarray chips containing 9,600 PCR-amplified cDNA fragments, 81 genes were down-regulated and 71 genes were up-regulated after curcumin treatment. Interestingly, below sublethal concentrations of curcumin (10 μM), several invasion-related genes were down-regulated, including: neuronal cell adhesion molecule (0.54-fold), matrix metalloproteinase 14 (MMP14; 0.65-fold), and integrins α6 (0.67-fold) and β4 (0.63-fold). In addition, several heat-shock proteins (Hsp) [Hsp27 (2.78-fold), Hsp70 (3.75-fold), and Hsp40-like protein (3.21-fold)] were up-regulated by curcumin treatment.

Using whole-genome microarrays, Nones *et al.* (*47*) investigated the effects of dietary curcumin on

colonic inflammation and gene expression in multidrug resistant gene-deficient ($mdr1a^{-/-}$) mice, a model of inflammatory bowel diseases (*47*). Microarray and pathway analyses suggested that the effect of dietary curcumin on colon inflammation could be mediated *via* an induction of xenobiotic metabolism and suppression of pro-inflammatory pathways, probably mediated by pregnane X receptor (Pxr) and peroxisome proliferator-activated receptor α (Ppara) activation of retinoid X receptor (Rxr). These results indicated the potential role of global gene expression and pathway analyses to study and better understand the effect of foods in modulating colonic inflammation (*47*).

Yan C *et al.* undertook a transcriptional profiling study to identify novel targets of curcumin. A cDNA array comprised of 12,625 probes was used to compare total RNA extracted from curcumin-treated and untreated MDA-1986 cells for differential gene expression. 202 up-regulated mRNAs and 505 down-regulated transcripts were identified. The proapoptotic activating transcription factor 3 (ATF3) was significantly up-regulated > 4-fold. In addition, two negative regulators of growth control [antagonizer of myc transcriptional activity (Mad) and p27kip1] were induced 68- and 3-fold, respectively. Furthermore, two dual-activity phosphatases (CL 100 and MKP-5), which inactivate the c-jun-NH$_2$-kinases, showed augmented expression, matching the reduced expression of the upstream activators of c-jun-NH$_2$-kinase (MEKK and MKK4). Of the down-regulated genes, the expression of Frizzled-1 (Wnt receptor) was strongly suppressed (8-fold). Additionally, two genes implicated in growth control (*K-sam*, encoding the keratinocyte growth factor receptor, and *HER3*) as well as the E2F-5 transcription factor, which regulates genes controlling cell proliferation, also showed decreased expression. Moreover, they identified activating transcription factor 3 (AFT3) as a novel contributor to the proapoptotic effect of curcumin (*48*). Sentrix Human WG-6 BeadChips were used to perform a large-scale gene-expression profiling during curcumin-triggered apoptosis (8-36 h) in follicular lymphoma HF4.9 cells (*49*). The comprehensive transcriptional response included differential expression of genes encoding apoptotic signaling proteins, transcription and splicing factors, tumor and metastasis suppressors, proteins involved in regulation of cell adhesion, lymphoid development, migration (*e.g.*, CXCR4) or B-cell activation (*e.g.,* CD20), and others (*49*).

In another study, to extend the knowledge on pathways or molecular targets already reported to be affected by curcumin (cell cycle arrest, phase-II genes) and to explore potential new candidate genes and pathways that could play a role in colon cancer prevention, a gene expression analysis in response to curcumin treatment was investigated in two human colon cancer cell lines (HT29 and Caco-2). Using cDNA microarrays containing four thousand human genes, HT29 colon cancer cells were exposed to two different concentrations of curcumin and gene expression changes were monitored in time (3, 6, 12, 24, and 48 h). Changes in gene expression after short-term treatment (3 or 6 h) with curcumin were also investigated in a second cell type, Caco-2 cells. Gene expression changes (> 1.5-fold) were observed at all time points. HT29 cells were more sensitive to curcumin than Caco-2 cells. Early response genes were involved in signal transduction, cell cycle, gene transcription, DNA repair, xenobiotic metabolism, and cell adhesion. A number of cell cycle genes, among them several that have a role in transition through the G$_2$/M phase, were modulated in HT29 cells after curcumin treatment. Furthermore, the observed changes in G$_2$/M cell cycle arrest genes were confirmed by flow cytometry. Moreover, they showed that some cytochrome P450 genes were downregulated by curcumin in both cell lines. In addition, curcumin affected expression of metallothionein genes, p53, tubulin genes, and other genes involved in colon carcinogenesis (*50*).

Ramachandran *et al.* compared the expression profiles of apoptotic genes induced by curcumin in the MCF-7 human breast cancer cell line and the MCF-10A mammary epithelial cell line (*51*). Microarray hybridization of Clontech apoptotic arrays which consisted of 214 apoptosis-associated genes was performed. Of the 214 apoptosis-associated genes, the expression of 104 genes was significantly altered after curcumin exposure. They reported that gene expression was altered up to 14-fold in MCF-7 as compared to MCF-10A (up to 1.5-fold) and concluded that the effect of curcumin was higher in MCF-7 cells compared to the MCF-10A mammary epithelial cell line. In MCF-7 cells, curcumin up-regulated 22 genes and down-regulated 17 genes. The up-regulated genes included CRAF1, GADD45, TRAF6, HIAP1, CASP2, CASP1, CASP3, CASP4, and TRAP3. The down-regulated genes included TNFR, TRIAL, PKB, and TNFRSF5 (*51*).

At the microRNA level, array analysis showed that curcumin alters the expression profiles of microRNAs in human pancreatic cancer cells. An oligonucleotide microarray chip was used to profile microRNA (miRNA) expression in pancreatic cells treated with curcumin. Curcumin altered miRNA expression in human pancreatic cells, up-regulating miRNA-22, and down-regulating miRNA-199a. They suggested that the biological effects of curcumin may be mediated through modulation of miRNA expression (*52*).

9. Artesunate and microarray

Artesunate (ART) is a semisynthetic derivative of artemisinin, the active principle of *Artemisia annua* L. ART and other artemisinin derivatives are novel drugs

in the treatment of malaria (*53*). Large clinical studies with malaria patients showed that ART is well tolerated, with insignificant side effects (*54*). In addition to the well known antimalarial activity of ART, we have previously identified a profound cytotoxic action of ART against cancer cell lines of different tumor types (*55*).

Previously, we identified mRNA expression profiles associated with the response of tumor cells to ART, Arteether, and Artemether (artemisinin derivatives) (*56*) and performed correlation and hierarchical cluster analyses of the inhibition concentration 50% (IC_{50}) values and mRNA expression levels of 464 genes deposited in the database of the National Cancer Institute (*57*). The mRNA expression of 208 out of 464 genes (45%) correlated significantly with IC_{50} values of at least one artemisinin derivative. These genes belong to different biological classes (oncogenes, apoptosis-regulating genes, drug resistance genes, tumor suppressor genes, DNA damage and repair genes, proliferation-associated genes and cytokines). Hierarchical cluster analysis identified two different gene clusters. One cluster contained genes which correlated significantly to all artemisinin derivatives. The second cluster contained genes differentially associated with the response of artemisinin derivatives to cancer cells.

Additionally, in order to confirm that Genome-wide microarray analyses provide an attractive approach to identify genes involved in the response of cancer cells to natural products, Anfosso *et al.* (*58*) investigated artemisinin and six derivatives and found a significant correlation of the sensitivity of these compounds to genes regulating tumor angiogenesis. Indeed, among mechanisms governing effects of artemisinins towards tumors is the inhibition of angiogenesis as shown by us and others (*59,60*).

Recently, we undertook a gene expression profiling study to identify novel molecular targets modulating the effect of artesunate on MiaPaCa-2 and BxPC-3 pancreatic cancer cell lines. cDNA microarray chips containing some 7,000 genes representing apoptotic, angiogenic, growth factors, anti-apoptotic, and metastasis-associated genes were used. Results showed that artesunate mediated growth inhibitory effects and induced apoptosis in pancreatic cancer cells through modulation of multiple signaling pathways. Moreover, we discovered that artesunate is a novel topoisomerase IIα inhibitor. Several molecular targets involved in the intrinsic and extrinsic apoptotic pathways were affected after treatment with ART. Among those molecular targets are *APAF1*, *BAX*, *BAK*, *CASP 2*, *CASP 3*, *CASP 4*, *CASP 5*, *CASP 6*, *CASP 8*, *CASP 9*, and *CASP 10*. Moreover, we have shown that the cytotoxic effect of ART on pancreatic cancer cells could be mediated, in part, through up-regulation of *DDIT3*, and *NAG-1* genes and down-regulation of *PCNA* and *RRM2* genes (*21*).

10. Cantharidin and microarray

Cantharidin is a vesicant product of Chinese blister beetles and Spanish flies (*61*). All body fluids of blister beetles have cantharidin, and the dried bodies have been used as an anticancer agent in traditional Chinese medicine for a long time (*62*). It was found that Cantharidin induces apoptosis of human multiple myeloma cells *via* inhibition of the JAK/STAT pathway (*63*). Zhang *et al.* (*64*) used cDNA microarrays (12,800 chip; United Gene Holdings, Ltd., PRC) to identify gene expression changes in HL-60 promyeloid leukemia cells treated with cantharidin. Cantharidin-treated cells decreased expression of genes coding for proteins involved in DNA repair (*e.g.*, *FANCG*, *ERCC*), DNA replication (*e.g.*, *DNA polymerase delta*), energy metabolism (*e.g.*, *isocitrate dehydrogenase alpha*, *ADP/ATP translocase*). Moreover, Cantharidin also decreased expression of genes coding for proteins that have oncogenic activity (*e.g.*, *c-myc*, *GTPase*) or show tumor-specific expression (*e.g.*, *phosphatidylinositol 3-kinase*). In addition, they suggested that cantharidin could be used as an oncotherapy sensitizer after reporting that exposure of HL-60 cells to cantharidin resulted in the decreased expression of multidrug resistance-associated protein genes (*e.g.*, *ABCA3*, *MOAT-B*). The increased expression of genes involved in modulating cytokine production and inflammatory response (*e.g.*, *NFIL-3*, *N-formylpeptide receptor*), partly explained the stimulating effects on leukocytosis (*64*).

In an attempt to identify key molecular determinants that involve sensitivity or resistance of tumor cells to cantharidin, we analyzed the microarray database of the National Cancer Institute (USA) in 60 tumor cell lines (*65,66*). Out of 9,706 genes identified, 21 genes whose mRNA expression correlated with the highest correlation coefficients to inhibition concentration 50% (IC_{50}) values were selected by COMPARE analysis and false discovery rate calculation (*66*). These genes were subjected to hierarchical cluster analysis to reveal whether the expression profiles of these genes could be used to predict sensitivity or resistance of cell lines to cantharidin. While the specific functions of the proteins encoded by the 21 identified genes were diverse, nevertheless, it is intriguing that many of them are involved in DNA repair, DNA damage response, and/or apoptosis (*66*).

11. Conclusion

DNA microarrays are a powerful and easy-to-use genomic tool. The genomic-wide data provided after microarray applications can provide potential information that helps to find the causes of disease, the mechanism of drug action, and the discovery of gene products that are targets for therapy in various diseases. With the use of this approach, novel molecular

targets and new therapeutic options can be identified. In this paper we have reviewed recent experiments using microarray technology involving medicinal plants derived from TCM as examples. Furthermore we showed that expression analysis was effective in identifying novel pathways and molecular targets mediating their effects.

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