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• BASIC RESEARCH •

Modulation of $GdCl_3$ and Angelica Sinensis polysaccharides on differentially expressed genes in liver of hepatic immunological injury mice by cDNA microarray

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Abstract

AIM: To study the modulating effect of $GdCl_3$ and Angelica Sinensis polysaccharides (ASP) on differentially expressed genes in liver of hepatic immunological mice by cDNA microarray.

METHODS: Hepatic immunological injury was induced by lipopolysaccharide (LPS ip, 0.2 mg·kg^{-1}) in bacillus calmetteguerin (BCG ip, 1 mg·kg^{-1}) primed mice; A single dose of 20 mg·kg^{-1} $GdCl_3$ was simultaneously pretreated and 30 mg·kg^{-1} ASP (ig, qd×7 d) was administrated when the BCG+LPS was primed. The mice were sacrificed at the end of the 7th day after ip LPS for 6 h and the liver was removed quickly. The PCR products of 512 genes were spotted onto a chemical material-coated glass plate in array. The DNAs were fixed to the glass plate after series of treatments. The total RNAs were isolated from the liver tissue, and were purified to mRNAs by Oligotex. Both mRNAs from the normal liver tissue and the liver tissue from the mice with hepatic immunological injury or that pretreated with $GdCl_3$ or ASP were reversely transcribed to cDNAs with the incorporation of fluorescent dUTP to prepare the hybridization probes. The mixed probes were hybridized to the cDNA microarray. After high-stringent washing, the cDNA microarray was scanned for fluorescent signals and showed differences between the two tissues.

RESULTS: Among the 512 target genes, 18 differed in liver tissue of hepatic immunological injury mice, and 6 differed in those pretreated by ASP, 7 differed in those pretreated by $GdCl_3$.

CONCLUSION: cDNA microarray technique is effective in screening the differentially expressed genes between two different kinds of tissue. Further analysis of those obtained genes will be helpful to understand the molecular mechanism of hepatic immunological injury and to study the intervention of drug. Both ASP and $GdCl_3$ can decrease the number of the differentially expressed genes in liver tissue of mice with hepatic immunological injury.

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INTRODUCTION

The advanced technique of DNA microarray makes it possible to monitor the expression of ten out of thousand genes simultaneously in one hybridization experiment[1,2]. The past year has demonstrated the versatility of microarrays for the analysis of whole model-organism genomes and has seen the development of chips to measure the expression of 40 000 human genes. Microarray technology has also become considerably more robust and sensitive[3]. The technology of microarrays has advanced from reverse Northern blots on filters detected using radioactive probes to a highly technical field involving miniaturized synthesis, multi-color fluorescent labeling, and database management. Recently, whole genome has been analyzed[4,5]. Microarray analyses typically follow the steps of gent selection, microarray synthesis, sample preparation, array hybridization, detection, and data analysis with appropriate controls required for each. Functional genomics is the study of gene function through the parallel expression measurements of genomes, most commonly using the technologies of microarrays and serial analysis of gene expression. Microarray could use in some other field also, such as, basic research of drug and target discovery, biomarker determination, pharmacology, toxicogenomics, target selectivity, development of prognostic tests and disease-subclass determination[6]. In this experiment, the DNA segments were spotted on a slide with high density. Then cDNA retro-transcribed from mRNA derived from normal or pathological tissues, which were labeled with Cy3 and Cy5 fluorescence, hybridized with the microarray slide[7]. Through this technique, detection of differentially expressed genes and the construction of differential gene expression profiles are greatly facilitated. The BioDoor 512DNA microarray was used for investigating the changes of gene expression in liver tissue of hepatic immunological mice and studying the effective mechanisms of ASP and $GdCl_3$, which might give important health benefits to understanding, diagnosing and treating the liver injury.

MATERIALS AND METHODS

Animals and treatments

Hepatic immunological injury was induced by lipopolysaccharide (LPS ip, 0.2 mg·kg^{-1}) in bacillus calmetteguerin (BCG ip, 1 mg·kg^{-1}) primed mice (Model); A single dose of 20 mg·kg^{-1} $GdCl_3$ was pretreated simultaneously (Model+$GdCl_3$) and 30 mg·kg^{-1} ASP (Model+ASP, ig, qd×7 d) was administrated when the BCG+LPS was primed. The mice were sacrificed at the end of the 7th day after ip LPS for 6 h and the liver was collected for analysis of cDNA micrarray.

Construction of microarrays[4]

The BioDoor 512 microarray consisted of a total of 512 novel or known genes (provided by United Gene Holdings, Ltd). These genes were amplified through PCR using universal primers and then purified; the purity of PCR production was dissolved in 3×SSC solution, and these target genes were spotted on silylated slides (Tele Chem, Inc.) by Cartesian 7 500 Spotting Robotics (Cartesian, Inc.). After spotting, the slides were hydrated (2 h), dried (0.5 h) at room temperature, UV cross-linked (65 mJ/cm), and then treated with 0.2 % SDS (10 min). The slides were dried again and ready for use.

Probe preparation[4]

The method of total RNA extraction was modified from the original single step extraction with Trizol agent. The liver tissue stored in liquid nitrogen were ground completely into tiny granules in 100 mm ceramic mortar (RNase free) and homogenized in Trizol. The pellet of total RNA was dissolved with Milli-Q H_2O. The mRNAs were purified using Oligotex mRNA Midi Kit (Qiagen, Inc.). The fluorescent cDNA probes were prepared through reverse transcription and then purified (referring to the protocol of Mark. Schena.). The probes from normal tissues were labeled with Cy3-dUTP, those from the pathological tissues were with Cy5-dUTP.

Hybridization and washing[4]

The probes were mixed and precipitated by ethanol, and resolved in 20 μl hybridization solution (5×SSC + 0.4 % SDS +50 % formamide + 5×denhardt's solution). Chip was prehybridized with Hyb sol +3 μl denatured salmon sperm DNA at 42 ℃ for 6 h. After denaturing at 95 ℃ for 5 min, the probe mixture was added on the prehybridized chip and covered with glass. The chips were inocubated at 42 ℃ for 15-17 h. The slide was then washed in solutions of 2×SSC + 0.2 % SDS, 0.1×SSC+ 0.2 % SDS and 0.1×SSC at 60 ℃, respectively for 10 min each, then dried at room temperature.

Detection and analysis[4]

The chip was scanned by ScanArray 5000 laser scanner (General Scanning, Inc) at 2 wavelengths. The acquired image was analyzed by ImaGene 3.0 software (BioDiscovery, Inc.). The intensity of each spot at the 2 wavelengths represented the quantity of Cy3-dUTP and Cy5-dUTP, respectively. The ratio of Cy3 to Cy5 was calculated. The two overall intensity was normalized by a coefficient according to the ratios of the located 40 housekeeping genes.

RESULTS

Differentially expressed gene in liver tissues between mice with or without hepatic immunological injury

Total 18 differentially expressed genes that might be associated with hepatic immnunological injury were selected from microarray. 4 genes showed up-regulation and 14 genes showed down-regulation (Table1, Figures 1 and 2).

Differentially expressed gene in liver tissues between mice with hepatic immunological injury and those pretreated by$GdCl_3$

6 differentially expressed genes associated with the effect of $GdCl_3$ on hepatic immnunological injury were selected from the microarray. 1 gene showed up-regulation and 5 genes showed down-regulation (Table 2, Figures 1 and 2).

Differentially expressed gene in liver tissues between mice with hepatic immunological injury and those pretreated by ASP

7 differentially expressed genes associated with the effect of ASP on hepatic immnunological injury were selected from the microarray. 1 gene showed up-regulation and 6 genes showed down-regulation (Table3, Figures 1 and 2).

Table 1 Differentially expressed gene in liver tissues between hepatic immunological injury mice and normal mice

Genbank-ID	Ratio(Cy5/Cy3)	Classification	Definition
Humipasa	0.253	Metabolism	Human mRNA for ATP synthase alpha subunit, complete cds.
Hsngmrna	0.285		H. sapiens hng mRNA for uracil DNA glycosylase.
Humoat	0.290	Modulators/Effectors/ intracellular transduction	Human ornithine aminotransferase mRNA, complete cds.
Hsncadhe	0.294	Cell Surface Antigens & Adhesion	Human mRNA for N-cadherin
Humpparp	0.307	0	Human acidic ribosomal phosphoprotein P0 mRNA, complete cds.
Humorf06	0.319	Other	Human mRNA for KIAA0106 gene, complete cds.
Hsgcsab	0.373	Modulators/Effectors/ intracellular Transduction	H. sapiens soluble guanylate cyclase small subunit mRNA.
Humhspa2	0.379	Stress Response Proteins	Human heat shock protein HSPA2 gene, complete cds.
Hspbx3	0.439	Other	Human PBX3 mRNA
Hsef1gmr	0.452		H.sapiens mRNA for elongation factor-1-gamma
Hsgagmr	0.460	Other	Human mRNA for GARS-AIRS-GART
Humhhr23	0.467	DNA Synthesis, Repair & Recombination F	Human mRNA for XP-C repair com;lamenting protein(p58/HHR23B), complete cds.
Hscatr	0.470	Metabolism	Human kidney mRNA for catalase.
Humscp2a	0.5	Ion Channel & Transport Proteins	Human sterol carrier protein X.sterol carrier protein 2 mRNA, complete cds.
Humnpm	2.062	Other	Human nucleophosmin mRNA, complete cds.
Hsrpl32	2.074	DNA Binding/Transcription/ transcription F	Human mRNA for ribosomal protein L32.
Hsu32944	2.244	Apoptosis-Related proteins	Human cytoplasmin dynein light chain1(hdlc1) mRNA, complete cds.
Hsu32944	3.239	Oncogenes & Tumor Suppressors mRNA, com;oete cds.	Human cytoplasmic dynein light chain 1 (hdlc1)

Ratio is Cy5/Cy3: high expression (ratio >2.0), low expression (ratio<0.5).

Table 2 Differentially expressed gene in liver tissues between mice with hepatic immunological injury and those pretreated by $GdCl_3$

Genbank-ID	Ratio(Cy5/Cy3)	Classification	Definition
Hsung	0.313	DNA Binding/Transcription/Tra nscription Factors	Human cDNA for uracil-DNA glycosylase.
Hsu09564	0.343	Modulators/Effectors/intracellular Transduction	Human serine kinase mRNA, complete cds.
Hsu83843	0.374	Cell Surface Antigens & Adhesion	Human HIV-1 Nef interacting protein (Nip7-1)mRNA, Partial cds.
Hsmyc1	0.441	Oncogenes & Tumor Suppressors	Human mRNA encoding the c-myc oncogene.
Humoat	0.454	Modulators/Effectors/intracellular transduction	Human ornithine aminotransferase mRNA, complete cds.
Hsu70323	2.200	Extracellular Cell signaling & Communication proteins	Human ataxin-2(SCA2) mRNA, complete cds.

Ratio is Cy5/ Cy3: high expression (ratio >2.0), low expression (ratio<0.5).

Table 3 Differentially expressed gene in liver tissues between mice with hepatic immunological injury and those pretreated by ASP

Genbank-ID	Ratio(Cy5/Cy3)	Classification	Definition
Humscp2a	0.283	Ion channel & transport proteins	Human sterol carrier protein X.sterol carrier protein 2 mRNA, complete cds.
Humoat	0.421	Modulators/Effectors/intracellular transduction	Human ornithine aminotransferase mRNA, complete cds.
Hsngmrna	0.431		H. sapiens hng mRNA for uracil DNA glycosylase.
humpafaa	0.461	Modulators/Effectors/ intracellular transduction	Human mRNA for platelet activating factor acetylhydrolase IB gamma-subunit, complete cds.
humhbgfb	0.481	Extracellular cell signaling & communication proteins	Human heparin-binding growth factor receptor (HBGF-R-alpha-a2) mRNA, complete ceds.
Hsu83843	0.493	Cell surface antigens and adhesion	Human HIV-1 Nef interacting protein(Nip7-1)mRNA, Partial cds.
Humnlk	2.353	Modulators/Effectors/intracellular transduction	Human neroleukin mRNA, complete cds.

Ratio is Cy5/ Cy3: high expression (ratio >2.0), low expression (ratio<0.5).

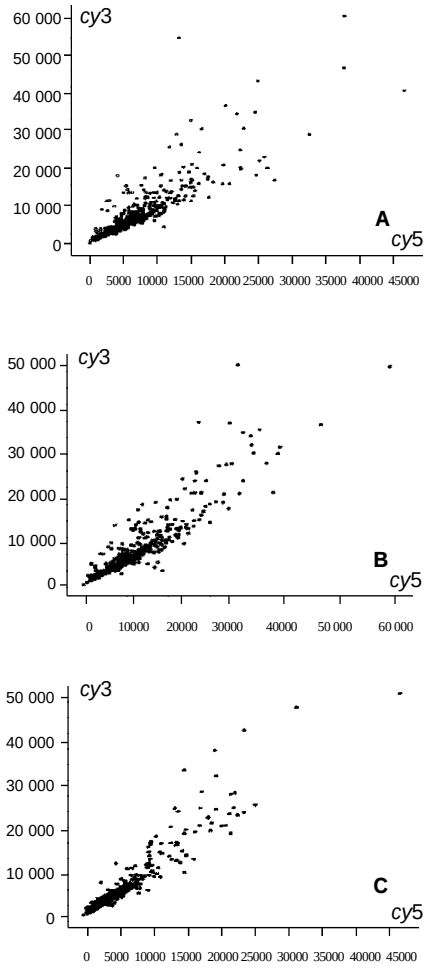

Figure 1 The scatter plots of gene expression pattern by cDNA microarray. A: Model liver(Cy5)/normal liver(Cy3); B: Model+$GdCl_3$ liver (Cy5)/normal liver; C: Mode+ASP liver (Cy5)/normal liver (Cy3). Ratio is Cy5/Cy3: high expression (ratio >2.0), low expression (ratio<0.5), no change in expression (0.5<ratio<2.0).

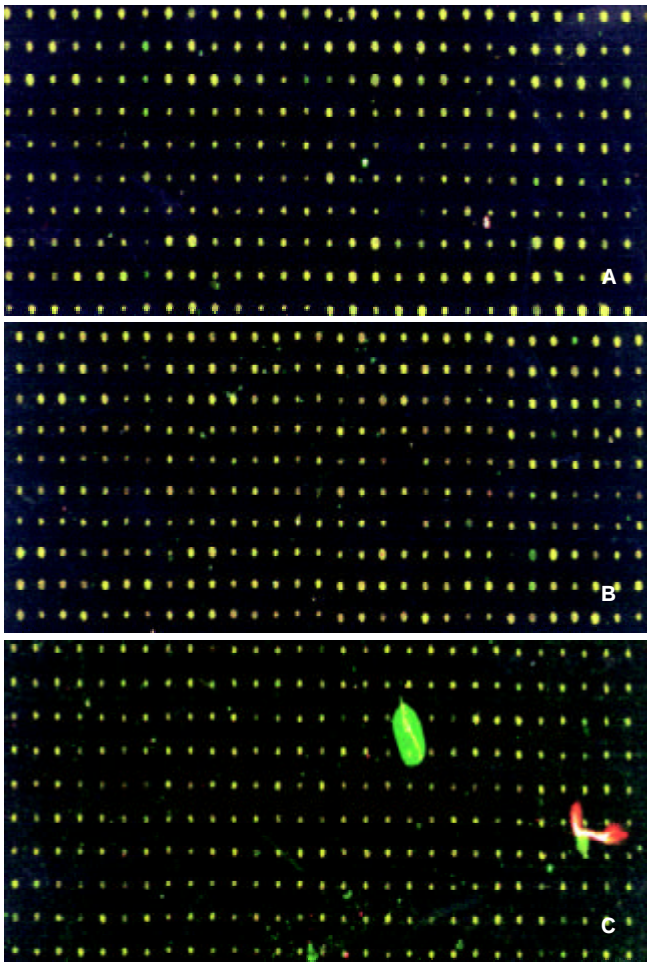

Figure 2 Scanning results of hybridizing signals by cDNA microarray. A: Model liver(Cy5)/normal liver(Cy3); B: Model+$GdCl_3$ liver (Cy5)/normal liver; C: Mode+ASP liver (Cy5)/normal liver (Cy3). Color of spots in the merged image of Cy3 image and Cy5 image: high expression (red), low expression (green), no change in expression (yellow).

DISCUSSION

Hepatic immunological injury is a result of altering multiple genes expression. cDNA microarray method can simultaneously determine a number of genes altered in the different pathological and physiological status, so gene chip is a useful tool for studying the target cause of disease and the effect of drug[10]. The DNA microarray hybridization applications reviewed include the important areas of gene expression analysis and genotyping for point mutations, single nucleotide polymorphisms (SNPs), and short tandem repeats (STRs)[11,12]. In addition to the many molecular biological and genomic research uses, systems for pharmacogenomic research and drug discovery[13,14], infectious and genetic disease and cancer diagnostics, and forensic and genetic identification purposes[15-17]. Additionally, microarray technology being developed and applied to new areas of proteomic and cellular analysis are reviewed[18,19]. Now, microarray has been developed many kinds, such as, protein microarray, GEM microarray, tissue microarray, they also have different kinds uses[20-22].

The present study showed that 18 differentially expressed genes that might be associated with hepatic immunological injury were found, 4 genes showed up-regulation and 14 genes showed down-regulation. These genes expression could recover to normal level by $GdCl_3$ except the HIV-1 Nef interacting protein (Nip7-1) mRNA (Hsu83843). And by ASP except the HIV-1 Nef interacting protein (Nip7-1) mRNA (Hsu83843), sterol carrier protein X/sterol carrier protein 2 Mrna (Humscp2a), and H.sapiens hug mRNA for uracil DNA glycosylase (Hsngmrna).

Furthermore, cDNA or uracil-DNA glycosylase, serine kinase mRNA, encoding the c-myc oncogene expression (Hsmycl) showed down-regulation in the mice pretreated with $GdCl_3$. In this study we find that using $GdCl_3$ and ASP to pretreat mice with hepatic immunological liver both would cause down-regulation of Hsung and Hsngmrna. Hsung and Hsngmrna both related to encoding uracil-DNA glycosylase. In humans, uracil appears in DNA at the rate of several hundred bases per cell each day as a result of misincorporation of deoxyuridine (dU) or deamination of cytosine. The human UNG-gene at position 12q24.1 encodes nuclear (UNG2) and mitochondrial (UNG1) two forms of uracil-DNA glycosylase[23]. Repair of uracil-DNA is a base-excision pathway initiated by a uracil-DNA glycosylase (UDG) enzyme of which have four families[24]. The most efficient and well characterized of these uracil-DNA glycosylases is UDG which releases uracil from DNA, restores the correct DNA sequence, excises U from single- or double-stranded DNA and is associated with DNA replication forks[25,26]. Uracil DNA glycosylase (UDG) and DNA polymerase beta (beta pol) are the two enzymes of base excision repair (BER)[10]. Krokan *et al* propose that BER is important both in the prevention of cancer and for preserving the integrity of germ cell DNA during evolution[23]. But Kvaløy *et al* find mutations affecting the function of human UNG gene are seemingly infrequent in human tumor cell lines[26,27]. So we infer down-regulation of these genes may affect cell proliferation, and $GdCl_3$ and ASP protect liver depend on this mechanism in some degree. Although ornithine aminotransferase mRNA (Humoat) also showed down-regulation, its expression showed a little improvement (Cy5/Cy3 increased from 0.290 to 0.454). Ataxin-2 Mrna (Hsu70323) expression showed up-regulation in the mice pretreated with $GdCl_3$. As for the mice pretreated with ASP, Humoat expression also showed some improvement (Cy5/Cy3 increased from 0.290 to 0.421). TNF alpha can cause liver cell apoptosis through the TNF-alpha receptor or Fas/CD95, which is expressed by liver cells. The TNF-alpha induced expression of the nuclear oncogene c-myc in intact hepatocytes has been studied[28]. The expression of Hsmycl is down-regulation, which may be the reason of preventing hepatic cell from apoptosis. The liver plays a central role in nitrogen metabolism. Nitrogen enters the liver as free ammonia and as amino acids of which glutamine and alanine are the most important precursors. Detoxification of ammonia to urea involves deamination and transamination. Half of the extra NH3 removed by the liver was, apparently, utilized by periportal glutamate dehydrogenase and aspartate aminotransferase for sequential glutamate and aspartate synthesis and converted to urea as the 2-amino moiety of aspartate[29]. Ornithine aminotransferase is the most important enzyme to metabolize free ammonia and amino acids. The gene exists as a single copy in the malarial genome and is located on chromosomes 6/7/8[30]. Ornithine-delta-aminotransferase (OAT) (EC 2.6.1.13) is a pyridoxal-5' phosphate dependent mitochondrial matrix enzyme. It controls the L-ornithine (Orn) level in tissues by catalysing the transfer of the delta-amino group of Orn to 2-oxoglutarate. The products of this reaction are L-glutamate-gamma-semialdehyde and L-glutamate[31]. Boon L proves that high protein diet would cause ornithine aminotransferase increased[32]. NOX significantly reduced serum levels of ornithine carbamoyltransferase and aspartate aminotransferase as hepatic injury[33]. The present study showed ornithine aminotransferase mRNA (Humoat) showed down-regulation, but its expression showed a little improvement (Cy5/Cy3 increased from 0.290 to 0.454) and (Cy5/Cy3 increased from 0.290 to 0.421) with $GdCl_3$ and ASP. So we think ornithine aminotransferase is important aspect in hepatic injury. Protective effect of $GdCl_3$ and ASP on the hepatic immunological injury depends on regulating the expression of ornithine aminotransferase. While the heparin-binding growth factor receptor (HBGF-R-alpha-a2) mRNA (humhbgfb) expression showed down-regulation and neuroleukin mRNA(Humnlk) expression showed up-regulation in the mice. Neuroleukin (NLK) is a multifunctional protein, involved in neuronal growth, glucose metabolism, cell motility, and differentiation. Neuroleukin (NLK), autocrine motility factor (AMF) and phosphohexose isomerase (PHI) have been identified same structure, so they have many kinds on functions and expressed in many tissues and organs, such as, brain, bone, liver, etc. NLK could mediate leukemia cell[34]. Romagnoli *et al* show that the block of NLK commits PC12 cells to caspase-dependent apoptosis, and suggest a general protective role of NLK in the control of cell death in neuronal cells[35]. NLK may be modulating inflammation and is probably involved in protecting CN and the cerebellums against apoptosis[36].When ASP pretreat mice with hepatic immunological injury, the expression of Humnlk up-regulation may show ASP prevent cell apoptosis. It remains to be demonstrated whether the altered genes are target of protective effect of $GdCl_3$ or ASP on the immunological liver injury, and further analysis the function of these genes is needed.

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