Expression profiling suggests a regulatory role of gallbladder in lipid homeostasis

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Abstract

AIM: To examine expression profile of gallbladder using microarray and to investigate the role of gallbladder in lipid homeostasis.

METHODS: 32P-labelled cDNA derived from total RNA of gallbladder tissue was hybridized to a cDNA array representing 17 000 cDNA clusters. Genes with intensities ≥2 and variation <0.33 between two samples were considered as positive signals with subtraction of background chosen from an area where no cDNA was spotted. The average gray level of two gallbladders was adopted to analyze its bioinformatics. Identified target genes were confirmed by touch-down polymerase chain reaction and sequencing.

RESULTS: A total of 11 047 genes expressed in normal gallbladder, which was more than that predicted by another author, and the first 10 genes highly expressed (high gray level in hybridization image), e.g., ARPC5 (2.225.88±90.46), LOC55972 (2.220.32±446.51) and SLC20A2 (1.865.21±98.02), were related to the function of smooth muscle contraction and material transport. Meanwhile, 149 lipid-related genes were expressed in the gallbladder, 89 of which were first identified (with gray level in hybridization image), e.g., FASN (11.42±8.90), APOD (92.61±8.90) and CYP21A2 (246.11±42.36), and they were involved in each step of lipid metabolism pathway. In addition, 19 of those 149 genes were gallstone candidate susceptibility genes (with gray level in hybridization image), e.g., HMGCR (10.98±0.31), NPC1 (34.88±12.12) and NR1H4 (16.8±0.65), which were previously thought to be expressed in the liver and/or intestine tissue only.

CONCLUSION: Gallbladder expresses 11 047 genes and takes part in lipid homeostasis.

INTRODUCTION

Cholesterol cholelithiasis is an extremely common, economically significant digestive disease that affects some 10-15% of the global population[1]. Gallstone is also the main cause of gallbladder carcinoma, biliary pancreatitis and iatrogenic lesions of the biliary tract. It has been reported that the USA spends 8-10 billion dollars on gallstone disease annually[2]. It was suggested from the data in the late 1980s that about 5.6% of the population in China was affected with gallstones[3], and the incidence may be increased in recent years. The patients with gallstone-related diseases hospitalized in the surgical department of our hospital accounted for 47% in 2001. Clearly, it is an important disease that deserves more attention.

Cholesterol saturated bile secreted by the liver is the prerequisite of gallstone formation, so liver is the place of lipid metabolism and becomes the focus of study. However, gallbladder is the place of stone formation and its relationship with lipid metabolism has been seldom investigated. Furthermore, the molecular mechanisms of gallstone formation in gallbladder-related with lipid metabolism are far from clear. Only about 40 genes (including gallstone susceptible gene loci) have been identified presently, and most of the previous studies were based on the changes in a single gene. Obviously, a total list of genes expressed in the gallbladder should be identified. The relatively new advent of cDNA array technology has provided a powerful method for large-scale expression profiling[4], and has led to the elucidation of a number of regulatory pathways involved in complex biological processes especially in tumor-related areas[5]. In this study, we used a powerful tool to examine expression profile of gallbladder and to investigate the role of gallbladder in lipid homeostasis. This would build a basis for understanding the physiological function of gallbladder, especially the mechanism of gallstone formation.
MATERIALS AND METHODS

cDNA array construction

cDNA clones were derived from liver and hepatocarcinoma cell lines, isolated from hypothalamus-pituitary-adrenal libraries[6] or purchased from Research Genetics (Huntsville, AL, USA). The assembled cDNA array contained 17 000 cDNA clones (representing the same number of independent cDNA clusters), of which 7 565 clusters were homologous to those found in the UniGene Database. All cDNA fragments were amplified and verified by electrophoresis. The average length of the cDNA fragments was -1 kb. PCR products were precipitated in isopropanol, redissolved in 10 µL of denaturing buffer (1.5 mol/L NaCl, 0.5 mol/L NaOH), and spotted on two 8 × 12-cm Hybond-N nylon membranes (Amersham Pharmacia, Buckinghamshire, UK) with an arrayer (BioRobotics, Cambridge, UK). Each spot carried -100 nL in volume and was 0.4 mm in diameter each cDNA fragment was placed in two different spots (double-offset). Lambda phage DNA and pUC18 vector DNAs were spotted as negative controls.

Hybridization intramembrane control

Eight housekeeping genes were used as hybridization intramembrane controls (HIC): ribosomal protein S9 (RPS9), β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase 1, M, 23 000 highly basic protein (RPL13A), ubiquitin C, phospholipase A2, and ubiquitin thiolesterase (UCHL1). These were evenly distributed in 12 places each per 8 × 12-cm array. Hybridization data was considered invalid if among the 12 spots representing the same gene, the intensity of the darkest spot exceeded 1.5-fold that of the weakest spot.

Clinical samples

Normal gallbladders were removed within 4 h postmortem from two adult males (aged 25 and 20 years, respectively) who died in traffic accidents. The Institute of Biomedical Science, Shanghai Second Medical University, approved the study, and all samples were obtained with informed consent. Tissues were frozen in liquid nitrogen immediately after separation, and kept frozen until used.

RNA extraction and probe preparation

Total RNA was extracted using a standard TRIzol RNA isolation protocol (Life Technologies, Inc., Grand Island, NY, USA). Poly (A)+ mRNA was then isolated from total RNA using a poly (dT) resin (Qiagen, Hilden, Germany). Approximately 1-2 µg of mRNA was labeled in a reverse transcription reaction in the presence of 200 µCi [α-32P] deoxyadenosine 5′-triphosphate (DuPont NEN, Boston, MA, USA) using Moloney murine leukemia virus reverse transcriptase as per the manufacturer’s instructions (Promega Corp., Madison, WI, USA).

Hybridization and image processing

Prehybridization was carried out in 20 mL of prehybridization solution (6× SSC, 0.5% SDS, 5× Denhardt’s, and 100 µg/mL denatured salmon sperm DNA) at 68 °C for 3 h. Overnight hybridization with the 32P-labeled cDNA in 6 mL of hybridization solution (6× SSC, 0.5% SDS, and 100 µg/mL salmon sperm DNA) was followed by stringent washing (0.1× SSC and 0.5% SDS at 65 °C for 1 h). Membranes were exposed to phosphor screens overnight and scanned using an FLA-3000A Plate/Fluorescent Image Analyzer (Fuji Photo Film, Tokyo, Japan). The radioactive intensity of each spot was linearly digitized to 65 536 gray-grade in a pixel size of 50 µm in an Image Reader, and recorded with Array Gauge software (Fuji).

Data collection and analysis

After subtraction of background values (±3) measured in an area where no cDNA was spotted, genes with intensities ≥2 were considered positive signals; this ensured that positives were distinguished from the background with a statistical confidence of >99.9%. Normalization among arrays was based on the sum of background-subtracted signals from all genes on the membrane[7]. The average hybridization intensities of two gallbladders were adopted to analyze the bioinformatics.

Touchdown reverse transcription polymerase chain reaction

For most of the genes, mRNA levels in the gallbladder samples were too low to be detected by standard dot blot and hybridization in situ, or even by conventional PCR, we used touch-down PCR to confirm the array hybridization results. All PCR products were verified by sequencing to avoid false positives.
RESULTS

Establishment of cDNA array system

Human cDNA clones randomly picked from cDNA libraries were terminally sequenced and compared with the Unigene database prior to their use in creating a cDNA array representing 17,000 genes or cDNA clusters (Figure 1). The reproducibility of the cDNA array analysis was evaluated in multiple replicated tests in which cDNA probes independently generated from the same mRNA sample were hybridized to different replicates of the cDNA arrays. The results from these experiments were almost perfectly concordant with a scatterplot $R^2$ (the square of the Pearson correlation coefficient, which measures similarity in gene expression patterns) of 0.97-0.98 (Figure 2). Of the 17,000 genes, only 0.2% showed >two-fold differences in their expression levels across different measurements. This showed that the cDNA array system was highly reproducible.

Gene list expressed in the gallbladder

In our work, a catalog of genes expressed in the human gallbladder was identified by cDNA array hybridization. The radio-intensities of corresponding spots on two parallel arrays were averaged. If the value was >2 and the variation <0.33 between the two samples, the signal was considered efficient. Of the 17,000 genes tested, a total of 11,047 genes were expressed in human normal gallbladder tissue, which is more than the number of 3,754 predicted by Lewis[8].

Top 10 genes expressed in the gallbladder

The 10 most highly expressed genes are listed in Table 1, and the top 3 breakpoint cluster region protein, uterine leiomyoma, 2; actin-related protein 2/3 complex, subunit 5 (16 ku) and eukaryotic translation initiation factor 4A, isoform 1, respectively.

Lipid metabolism-related genes and gallstone candidate genes in gallbladder

Totally 149 lipid metabolism-related genes were expressed in the gallbladder (Table 2). Eighty-three of them were identified for the first time in gallbladder (results after searching Unigene and Pubmed). Lammert et al have listed 45 possible gallstone candidate genes based on previous knowledge, 24 of which were assembled in our array, and 19 of these 24 genes were lipid-related genes and expressed in the gallbladder. We selected four lipid-related genes randomly, and by touchdown reverse transcription polymerase chain reaction (RT-PCR) and sequencing, confirmed the results in cDNA array (Figure 3).

Table 1 The top 10 highly expressed genes in normal gallbladder tissue

| Name of gene                                    | Symbol | Gray level (mean±SD) |
|-------------------------------------------------|--------|----------------------|
| Breakpoint cluster region protein, uterine leiomyoma, 2 | BCRP2  | 2.738.74±23.23       |
| Actin-related protein 2/3 complex, subunit 5 (16 ku) | ARPC5  | 2.225.88±90.46       |
| Eukaryotic translation initiation factor 4A, isoform 1 | EIF4A1 | 2.225.86±274.74      |
| Mitochondrial carrier family protein            | LOC5972| 2.220.32±446.51      |
| Solute carrier family 20 (phosphate transporter), member 2 | SLC20A2 | 1.865.21±98.02      |
| Cytochrome b5 reductase 1 (B5R1)                | LOC51706| 1.851.01±598.45     |
| ADP-ribosylation factor 1                       | ARF1   | 1.844.63±533.31      |
| FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived) | FARP1  | 1.654.95±126.85      |
| Proteasome (prosome, macropain) activator subunit 2 (PA28 beta) | PSME2  | 1.635.33±148.15      |
| cAMP responsive element binding protein-like 1  | CREB1  | 1.579.36±71.68       |

Figure 3 RT-PCR and sequencing confirmation of genes that were expressed in the gallbladder. RT-PCR electrophoresis result: A: CYP27A1 (320 bp); B: NR1H4 (352 bp); C: CMOAT (353 bp); D: AKR1C3 (240 bp). Sequencing result of PCR product: E: CYP27A1; F: CMOAT.
### Table 2 Lipid-related genes expressed in normal gallbladder

| GenBank ID | Gene name | Symbol | Reported or not | Gray level (mean±SD)          |
|------------|-----------|--------|-----------------|--------------------------------|
| NM_000859  | 3-Hydroxy-3-methylglutaryl-Coenzyme A reductase | HMGCR | P\(^i\)          | 10.98±0.31                     |
| U66669     | 3-hydroxyisobutyryl-Coenzyme A hydrolase       | HIRCH | P\(^i\)          | 11.17±0.09                     |
| U29344     | Fatty acid synthase                            | FASN  |                 | 11.42±2.62                     |
| AA469091   | ATPase, aminophospholipid transporter-like, Class I, type 8A, member 2 | ATP8A2 |                 | 11.53±1.08                     |
| BE730527   | Lipase protein                                  | LOC574A0 | P\(^i\) | 11.58±0.85                     |
| AL043165   | Homolog of mouse transient receptor potential-phospholipase | LTRPC7 |                 | 11.59±0.69                     |
| U22662     | Nuclear receptor subfamily 1, group H, member 3  | NRH13  |                 | 11.77±0.85                     |
| M14564     | Cytochrome P450, subfamily XVII (sterol 17-alpha-hydroxylase), adrenal hyperplasia | NR1H3  |                 | 11.77±0.85                     |
| NM_0010844 | Solute carrier family 21 (organic anion transporter), member 8\(^i\) | SLC21A8 | P\(^i\)          | 11.14±0.77                     |
| AA469091   | ATPase, aminophospholipid transporter-like, Class I, type 8A, member 2 | ATP8A2 |                 | 11.53±1.08                     |
| BE730527   | Lipase protein                                  | LOC574A0 | P\(^i\) | 11.58±0.85                     |
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| Gene Name | Description | Expression Value |
|-----------|-------------|------------------|
| U68233   | Nuclear receptor subfamily 1, group H, member 4** | 16.80±0.65 |
| AF035284 | Fatty acid desaturase 1 | 16.82±0.92 |
| X04898   | Apolipoprotein A-II | 16.90±0.60 |
| AL031295 | LysophospholipaseII | 17.01±0.19 |
| M55150   | Fumarylacetoacetate hydrolase (fumarylacetoacetase) | 17.41±2.04 |
| M31210   | Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1 | 17.56±0.62 |
| BE395256 | Lanosterol synthase | 17.63±1.71 |
| NM_000483| Apolipoprotein C-II | 17.71±1.70 |
| AL031295 | 3-Hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (hydroxymethylglutaricaciduria) | 17.86±0.79 |
| BE714757 | Lipase A, lysosomal acid, cholesterol esterase (Wolman’s disease) | 18.21±2.02 |
| AF077820 | Oxidised low density lipoprotein (lectin-like) receptor 1 | 18.73±0.37 |
| X01388   | Cytochrome P450, subfamily XIB (steroid 11-beta-hydroxylase), polypeptide 1 | 19.12±3.33 |
| AL034374 | Homolog of yeast long chain polyunsaturated fatty acid elongation enzyme | 20.63±1.82 |
| AF065215 | Phospholipase A2-activating protein | 20.73±4.28 |
| AB006746 | Phospholipid scramblase 1 | 21.33±0.62 |
| M59979   | Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | 21.70±0.75 |
| X12162   | Apolipoprotein A-I | 21.70±0.75 |
| AF079167 | Oxidised low density lipoprotein (lectin-like) receptor 1 | 21.70±0.75 |
| U55764   | Sulfotransferase, estrogen-preferring | 22.12±4.28 |
| J03459   | Leukotriene A4 hydrolase | 22.32±4.28 |
| X67696   | Niemann-Pick disease, type C2 gene | 22.32±4.28 |
| AL049748 | Apolipoprotein L, 5 | 22.61±1.71 |
| X51416   | Estrogen-related receptor alpha | 22.81±2.02 |
| AF056215 | Phospholipase A2, group IBV (cytosolic) | 23.00±1.84 |
| AB006746 | Phospholipid scramblase 1 | 23.00±1.84 |
| M59979   | Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | 23.00±1.84 |
| X05764   | Cytochrome P450, subfamily XIB (steroid 11-beta-hydroxylase), polypeptide 1 | 23.00±1.84 |
| AL022318 | Phorbolin (similar to apolipoprotein B mRNA editing protein) | 23.00±1.84 |
| X12162   | Apolipoprotein C-I | 23.00±1.84 |
| NM_001645| Apolipoprotein E | 23.00±1.84 |
| X47471   | Cytochrome P450, subfamily XII (steroid 11-beta-hydroxylase), polypeptide 2 | 23.00±1.84 |
| X13318   | Apolipoprotein C-III | 23.00±1.84 |
| R98624   | Bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase) | 23.00±1.84 |
| U93305   | Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | 23.00±1.84 |
| AL022318 | Phorbolin (similar to apolipoprotein B mRNA editing protein) | 23.00±1.84 |
| X07228   | Lipase, hepatic | 23.00±1.84 |
| AL013200 | Glycosylphosphatidylinositol specific phospholipase D1 | 23.00±1.84 |
| AA126778 | Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) | 23.00±1.84 |
| Z99390   | L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain | 23.00±1.84 |
| AF002225 | Niemann-Pick disease, type C1 | 23.00±1.84 |
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DISCUSSION

A cDNA array representing 17 000 human genes or cDNA clusters was established. Compared with the cDNA array without hybridization intra membrane controls, the HIC in the cDNA array system significantly contributed to the evenness of hybridization among different parts of the array membrane and therefore improved the reliability of the array analysis (data not shown). Replicated examinations of the same sample indicated that only 0.2-0.3% of the genes spotted or 0.5% informative genes, might be false-positive signals. Because we chose the genes that were lower than 0.33 in the variation of average on two samples, the possibilities of false-positives were remote.

Totally 11 047 genes were expressed in the gallbladder, which is almost thrice the number of 3 754 predicted by the ADORA2BP adenosine A2b receptor LIKE pseudogene, the IRF6 gene for interferon regulatory factor 6 and two novel possibilities of false-positives were remote. Because we chose the genes that were lower than 0.33 in the variation of average on two samples, the possibilities of false-positives were remote.

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demonstrates that the gallbladder takes part in lipid homeostasis. Those genes were expressed in the gallbladder, too, suggesting gallbladder takes part in both digestion and lipid homeostasis. Those are displayed in Table 1, they are: BCRP2: breakpoint cluster region protein, uterine leiomyoma, 2; ARPC5: actin-related protein 2/3 complex, subunit 5 (16 ku); EIF4A1: eukaryotic translation initiation factor 4A; ARF1: ADP-ribosylation factor 1; FARPI: FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived); PSME2: proteasome (prosome, macropain) activator subunit 2 (PA28 beta); CREBL1: cAMP responsive element binding protein-like 1. Their functions are associated with smooth muscle contraction and material transport, thus, they may participate in the contraction and concentration of gallbladder bile[9-16].

The human body contains both exogenous and endogenous cholesterol. Exogenous cholesterol comes from diet while the endogenous is synthesized inside the body by liver cells. Excess total cholesterol in the plasma will eventually become cholesterol. Exogenous cholesterol comes from diet while current a labor-consuming and accurate work, but it needs complex procedures, and can be affected by many factors, thus, it is not so sensitive. On the contrary, cDNA array is a high-throughput and relatively less expensive technology.

The top 10 highly expressed genes with a clear function are displayed in Table 1, they are: BCRP2: breakpoint cluster region protein, uterine leiomyoma, 2; ARPC5: actin-related protein 2/3 complex, subunit 5 (16 ku); EIF4A1: eukaryotic translation initiation factor 4A; isof orm 1; LOC55972: mitochondrial carrier family protein; SLIC20A2: solute carrier family 20 (phosphate transporter), member 2; LOC51706: cytochrome b5 reductase 1 (B5R.1); ARF1: ADP-ribosylation factor 1; FARPI: FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived); PSME2: proteasome (prosome, macropain) activator subunit 2 (PA28 beta); CREBL1: cAMP responsive element binding protein-like 1. Their functions are associated with smooth muscle contraction and material transport, thus, they may participate in the contraction and concentration of gallbladder bile[9-16].

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