Expressed genes in regenerating rat liver after partial hepatectomy

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Abstract

AIM: To reveal the liver regeneration (LR) and its control as well as the occurrence of liver disease and to study the gene expression profiles of 551 genes after partial hepatectomy (PH) in regenerating rat livers.

METHODS: Five hundred and fifty-one expressed sequence tags screened by suppression subtractive hybridization were made into an in-house cDNA microarray, and the expressive genes and their expressive profiles in regenerating rat livers were analyzed by microarray and bioinformatics.

RESULTS: Three hundred of the analyzed 551 genes were up- or downregulated more than twofolds at one or more time points during LR. Most of the genes were up- or downregulated 2-5 folds, but the highest reached 90 folds of the control. One hundred and thirty-nine of them showed upregulation, 135 displayed downregulation, and up or down expression of 26 genes revealed a dependence on regenerating livers. The genes expressed in 24-h regenerating livers were much more than those in the others. Cluster analysis and generalization analysis showed that there were at least six distinct temporal patterns of gene expression in the regenerating livers, that is, genes were expressed in the immediate early phase, early phase, intermediate phase, early-late phase, late phase, terminal phase.

CONCLUSION: In LR, the number of down-regulated genes was almost similar to that of the upregulated genes; the successively altered genes were more than the rapidly transient genes. The temporal patterns of gene expression were similar 2 and 4 h, 12 and 16 h, 48 and 96 h, 72 and 144 h after PH. Microarray combined with suppressive subtractive hybridization can effectively identify the genes related to LR.

INTRODUCTION

In the healthy adult rat liver, most of the hepatocytes lie in G0 phase, and their cell division index is very low (about one ten thousandth). However, metabolism of hepatocytes is quickly altered after partial hepatectomy (PH). Activation of hepatocytes in G0 phase occurs about 2 h after PH, and they progress to G1 phase about 6 h after PH. Then, the cells enter into S phase of cell cycle in 12 h. DNA synthesis occurs in the early 6 h (12-17 h) of S phase, and then DNA is synthesized 18-30 h after PH, which reaches a maximum at 24 h. The G2 phase of cell cycle lies in the subsequent 2-4 h (31-34 h after PH). After that, hepatocytes go on dividing, and the peak of cell division is at 36 h after PH. The next cycle of hepatocytes is in the following 36-66 h after PH. The re-differentiation of liver cells and the re-building of regenerated livers are in 72-144 h after PH. Many experiments have confirmed that a cell cycle of hepatocytes lasts for about 30 h, but that of other cells distinguishes from them. Briefly, cells in the residual liver would be activated to proliferate, re-differentiate and rebuild their structure and function after PH. In different phases of liver regeneration (LR), the physiological and biochemical actions of different kinds of cells in the liver are different. The categories and amounts of the expressed genes in them are various. To learn the molecular mechanism of LR, it is essential to highlight how many genes are related to it. Therefore, this paper reports that 300 genes have been successfully identified to correlate with LR by combing microarray in combination with suppression subtractive hybridization.

MATERIALS AND METHODS

Partial hepatectomy of rats

Healthy SD rats weighing 200±20 g were obtained from the Experimental Animal Center of Henan Normal University. Following the method of Higgins and
Anderson[18], 70% of the rat liver was removed under sterile conditions.

**Regenerating liver preparation and RNA isolation**

The regenerating livers of four rats (male:female = 1:1) were taken 2, 4, 8, 12, 16, 24, 36, 48, 72, 96 and 144 h after PH. The livers were rinsed in cold PBS and immersed in a -80 °C refrigerator for RNA extraction. Total RNA was isolated from frozen livers according to the manual of TRIzol kit of Invitrogen. In brief, 50-100 mg liver was homogenized in 1 mL TRIzol reagent containing phenol and guanidinium isothiocyanate/cationic detergent, followed by phenol-chloroform extraction and isopropyl alcohol precipitation. The quantity and integrity of total RNA were examined by an ultraviolet spectrometer and denaturing formaldehyde agarose electrophoresis by ethidium bromide staining.

**Subtracted cDNA library construction and screening**

cDNA subtracted libraries were generated from total RNA by PCR-Select™ cDNA subtraction kit (Clontech) following the manufacturer’s instructions. Briefly, total RNA was transcribed into double cDNA strands and digested with restriction enzymes, followed by subtracted hybridization with drivers and testers. Finally, differential expression sequence tags were performed to construct subtracted cDNA libraries with suppression PCR.

**Sequence analysis**

Base sequence assay of ESTs was carried out according to the current protocols in molecular biology. All sequences were determined on both strands. Comparison analysis of the selected sequences was conducted with the DNAman and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) GenBank database[17].

cDNA microarray construction

cDNA fragments amplified by PCR with nested PCR primer1 and primer2 and purified by NaAc/isopropyl alcohol were spotted onto glass slides (BioStar) with the help of the ProSys-5510A spotting machine. Then the gene chips were ready by hydrating and blocking and drying. A total of 1 152 elements (double spot chip) including 50 control system genes (8 negative control, 12 void control, 30 internal control) and 551 target genes to be studied within 6 h and then recovered gradually to the control levels downregulated, and 10 were either up- or downregulated in 48-72 h after PH. In the cell cycle progression phase of LR, the expression of 34 genes was rapidly altered different phases of LR. Following biochemical actions, the 133 elements made in microarray, the expression of the hybridization results was performed for green signals (downregulation), yellow signals (no obvious regulation), and red signals (upregulation)[17].

**Hybridization and scanning**

Glass slices were prehybridized at 42 °C for 5-6 h in hybridization buffer containing freshly cooked salmon sperm DNA. The labeled denatured probes were hybridized against cDNA microarray and incubated overnight (16-18 h) at 42 °C. The slices were then washed twice with 2× SSC containing 0.5% SDS for 5 min at room temperature, then with 0.2× SSC containing 0.5% SDS at 60 °C for 10 min, and finally with 0.2× SSC at 60 °C for 10 min. The slices were exposed to a photographer. Hybridized images were scanned by a fluorescence laser-scanning device, GenePix4000A. At least, two hybridizations were performed at each time point. In addition, a semiquantitative inspection of the hybridization results was performed for green signals (downregulation), yellow signals (no obvious regulation), and red signals (upregulation)[17].

**Data analysis**
cy3 and cy5 signal intensities were quantified by GenePixPro 3.0 software. Subsequently, we normalized the obtained numerical data with classical linear regression techniques. In brief, quantified cy3 and cy5 signal intensities were obtained when foreground signal intensities were deducted by background signal intensities and cy5 signal intensities were replaced by 200 when they were <200. When Ri (Ri = cy5/cy3) was between 0.1 and 10, Ri was taken as logarithms to generate R’i [log(Ri)] and ND was taken by EXP (R) (averaged R’i). The modified cy3* was generated by ND multiplying cy3 and was replaced by 200 when it was <200. The ratio was expressed by cy5/cy3*. Therefore, we selected the genes, whose ratio was more than 2 or less than 0.5 representing a twofold difference in expression level. To analyze the selected gene expression data, we performed GeneMaths cluster analysis and hierarchical clustering to appraise the number of groups. Whole analyses were executed using Microsoft Excel and GeneSpring[11,17].

**RESULTS**

The analysis of gene expression spectrum showed that, of the 551 elements made in microarray, the expression intensity of 300 genes increased or decreased at least more than twofolds at one time point after PH. Their sequence analysis showed that 152 were unreported genes, and 133 genes were significantly reported. Of which 49 were upregulated in regenerating rat livers, 74 were downregulated, and 10 were either up- or downregulated in different phases of LR. Following biochemical actions, the 133 elements were categorized into 24 groups (Table 1).

Analysis of their expression in the regenerating livers at different time points after PH showed that, in the priming phase of LR, the expression of 34 genes was rapidly altered within 6 h and then recovered gradually to the control levels in 48-72 h after PH. In the cell cycle progression phase of 8-36 h after PH, the expression of 86 genes was altered markedly. In the terminal phase of LR over 72 h after PH, the expression of 13 genes was altered distinctly (Figure 1).

Clustering analysis of genes expressed at 12 time points after PH showed that the 300 elements altered at least more than twofolds in density at one time point (Figure 2A), and...

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| No. | Gene description | Fold difference | No. | Gene description | Fold difference |
|-----|------------------|-----------------|-----|------------------|-----------------|
| 29 | Solute carrier family 20 (phosphate transporter) member 1 (Slc20a1) | 3.3, 0.1 | 68 | UTP-glucose-1-phosphatase | 0.3 |
| 28 | Cytochrome P450 2E1 | 7.0 | 69 | Angiopoietin-like 3 | 0.2 |
| 27 | Cytochrome P450 15-beta (Cyp2c12) | 2.1, 0.3 | 70 | Ring1 and Y11 binding protein | 0.5 |
| 26 | Cytochrome P450 15-beta | 2.3, 0.5 | 71 | Chromatin remodeling factor | 2.0 |
| 25 | Cytochrome P450 0.2 | 2.2 | 72 | Hemooglobin | 0.3 |
| 24 | P450 arachidonic acid epoxygenase (cyp 2C23) | 0.2 | 73 | Immunological proteins | 0.3 |
| 23 | Peroxisomal beta-hydroxyacyl-CoA oxidase (Perox) | 0.2 | 74 | 11beta-hydroxysteroid dehydrogenase | 0.3 |
| 22 | Malonyl-CoA decarboxylase | 0.3 | 75 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.4 |
| 21 | Cytochrome P450 cholesterol 7-alpha-hydroxylase (Cyp7alpha) | 2.3, 0.5 | 76 | Amyloid P-component (Sap) | 0.4 |
| 20 | Prostaglandin D2 synthase 2 (Ptgds2) | 3.0 | 77 | Amyloid alpha-5 protein | 0.2 |
| 19 | Methylmalonate semialdehyde dehydrogenase | 0.3 | 78 | Cocoa protein | 2.1 |
| 18 | 3-alpha-hydroxysteroid dehydrogenase | 0.2 | 79 | Probable surface antigen protein | 2.5 |
| 17 | Alpha-1 major acute phase protein | 6.2 | 80 | Dna1 (Dna1p) | 2.1 |
| 16 | Acute-phase protein alpha-1-inhibitor | 2.4 | 81 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 15 | Interferon-induced protein with tetratricopeptide repeats | 0.2 | 82 | Clathrin, heavy polypeptide (Clta1) | 3.3 |
| 14 | Methylalpha-1, 6-glycoprotein beta-1, 2.0 | 0.3 | 83 | Arginase 1 (Arg1) | 0.4 |
| 13 | NAD(P)-dependent steroid dehydrogenase | 0.4 | 84 | Mitochondrial adenine nucleotide | 5.3 |
| 12 | 3-alpha-hydroxysteroid dehydrogenase | 0.2 | 85 | Ribosomal protein S19 | 2.5 |
| 11 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.3 | 86 | Ribosomal protein L41 (Rpl41) | 2.1 |
| 10 | 3-alpha-hydroxysteroid dehydrogenase | 0.2 | 87 | Ribosomal protein L41 (Rpl41) | 2.1 |
| 9 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.3 | 88 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 8 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.4 | 89 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 7 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.5 | 90 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 6 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.6 | 91 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 5 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.7 | 92 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 4 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.8 | 93 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 3 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 0.9 | 94 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 2 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 1.0 | 95 | Ribosomal protein L28 (Rpl28) | 2.2 |
| 1 | 2-hydroxyphytanoyl-CoA lyase (Hpcl2) | 1.1 | 96 | Ribosomal protein L28 (Rpl28) | 2.2 |

Table 1 Genes altered at least by more than twofold intensity at one time point after PH
| Gene Name                                                                 | Fold Change (2-24 h) | function                                                                 |
|---------------------------------------------------------------------------|----------------------|--------------------------------------------------------------------------|
| Interleukin 1 receptor, type I (Il1r1)                                     | 7.9                  | 120 ATP synthase alpha subunit (Atp5a1)                                 |
| Golgi SNAP receptor complex member 1 (Gosr1)                               | 0.4                  | 121 Bifunctional aminosacyl-tRNA synthetase (Aars)                       |
| Nuclear receptor subfamily 0, member 2 (Nr0b2)                             | 0.2                  | 122 ATP synthase subunit 8                                              |
| Lyosomal-associated protein transmembrane 4A (Lypa4)                       | 0.5                  | 123 Glutamyl-prolyl-tRNA synthetase (Eprn)                               |
| ATP-binding cassette, sub-family B, member 4 (Atp5a1)                      | 0.4                  | 124 Fatty acid elongase 1 (Elo1)                                        |
| ATP-binding cassette, sub-family C, member 1 (Atphb1)                      | 0.2                  | 125 RNA cyclase                                                         |
| Golgi SNAP receptor complex member 1 (Gosr1)                               | 0.4                  | Transferrases                                                           |
| Nuclear receptor subfamily 0, member 2 (Nr0b2)                             | 0.2                  | 126 Camitine 0-octanoyltransferase (Crot)                               |
| Early growth response factor 1 (Egr1)                                     | 3.6                  | 127 Glutathione S-transferase, alpha 1 (Gsta1)                          |
| Neuropeptide Y (Npy)                                                      | 18.2                 | 128 Glutathione S-transferase, type 3 (Gst3)                            |
| NF-E2-related factor 2 (Nfe2l2)                                           | 0.4                  | 129 Microsomal glutathione S-transferase 1 (Gstl1)                      |
| Pre-B-cell colony-enhancing factor (Pbef)                                  | 3.5                  | 130 Sulfotransferase K2                                                |
| Amphoterin                                                                | 0.3                  | 131 Sialyltransferase 1 (Stat1)                                         |
| Neuropeptide Y (Npy)                                                      | 18.2                 | 132 UDP-glucuronosyltransferase member (Ugt2b5)                          |
| Insulin-like growth factor I                                               | 0.5                  | 133 Protein disulfide isomerase-related protein                         |

**Graphs:**

- 2-8 h, 2-12 h
- 2-24 h
- 2-36 h
- 2-48 h
- 2-72 h, 2-96 h, 2-144 h
- 2-48 h
- 8-16 h, 8-24 h
- 8-36 h
- 8-72 h, 8-144 h
- 12-16 h.
that the most similar patterns of gene expression were located next to each other and placed in a major branch of the dendrogram. Twelve and sixteen hours 48 and 96 h, 72 and 144 h patterns were clustered as separate groups in major branches, indicating that these time points shared common expression profiles of genes (Figure 2B). Thirty-four genes were induced to express in 2-4 h and reached a maximum in 8-24 h, but a smaller peak of gene expression appeared at 72 h after PH. To facilitate the visualization and interpretation of the gene expression program in these data, we used the method of GeneMaths to order genes of similarities in their expression patterns and displayed them.

Figure 1 Gene expression differences at different time points after PH. The 133 elements were categorized into 21 temporal patterns following the induction of suppressed time points.

Figure 2 Hierarchical cluster analysis of 300 elements. A: Cluster of distribution trend. Three hundred elements differing by at least more than twofold intensity at one time point were identified; B: Cluster of hierarchical relativity. A hierarchical cluster of 11 time points showed that 12 and 16 h, 48 and 96 h as well as 72 and 144 h patterns were clustered together as separate groups in major branches.
Based on the expressed characteristics of genes in LR and following the review of Michalopoulos and Defrances, the selected elements were categorized into six distinct temporal patterns of expression: the genes of rapid induction which were expressed in the immediate early phase of 2-6 h after PH, the genes of early induction in the early phase of 8-16 h, the genes of middle induction in the intermediate phase of 16-24 h, the genes of early-late induction in the early-late phase of 24-36 h, the genes of late induction in the late phase of 36-72 h, and the genes of consistent repression in the terminal phase of 72-144 h.

It was found that expression of the 133 genes in the regenerating livers was quite different. Seventy-four of them decreased 2-10 folds, 49 of them were increased to 2-5 folds, 9 genes to 5-10 folds, 3 genes to over 10 folds, and the maximum to over 90 folds (Figure 4).

**DISCUSSION**

**Genes of rapid induction expression**

In the immediate early phase of LR (2-6 h after PH), liver damage occurred in inflammation response, hepatocytes in G₀ phase were activated, and then progressed to G₁ phase.

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**Figure 3** Cluster analysis of differentially expressed genes identified by cDNA microarray-based gene expression profiling. These genes were classified into 12 clusters by GeneMaths.
Thirty-four genes, except the previous reported genes, were consistently and rapidly induced to express. 19 of them were upregulated and 15 downregulated. Among the genes, NF-E2-related factor 2 (Nfe2l2), isocitrate dehydrogenase, JE/MCP-1, and complement component 5 were advantageous to block oxidative injuries and to prevent the occurrence of inflammation of the residual liver after PH. Pre-B-cell colony-enhancing factor (PBEF), nuclear protein 1 (Nupr1), early growth response factor 1 (Egr1) were upexpressed, which probably play an important role in activating hepatocytes to start DNA synthesis[18]. The expression of isozymes of aspartate aminotransferase and phosphodiesterase 1 (Enpp1) was induced, which supply purine and adenosine for DNA synthesis[19]. The proteins of providing energy for LR such as adipose differentiation-related protein and phosphatidylserine-specific phospholipase A1 and proteins associated with inflammation response and cell apoptosis such as α1 major acute phase protein, fibrinogen gamma polypeptide (Fgg), kininogen and class III Fe-gamma receptor were also included, which play important parts in preventing the occurrence of inflammation and the startup of LR in the immediate early phase.

Initiated genes in the early phase of LR

In the early phase of LR (8-16 h after PH), livers damaged stress response and hepatocytes were prepared to synthesize DNA. The expression of 31 genes was altered at 8 h after PH. Among them, 11 were upregulated and 20 downregulated, of which, 5 genes reached expression peak at 8 h after PH. Myelin-associated glycoprotein (MAG) was induced, which is important for nerve system regeneration in LR[20]. The stress-inducible 70-kd protein (Hsp70) and protein disulfide isomerase-related protein were expressed, which promote protein folding correctly and degradation of harmful proteins in LR. Serum amyloid P component and clusterin were repressed in 8-16 h after PH, which can decrease accumulation of false or toxic proteins in neurocytes, and are useful in protecting neonatal livers[21]. Plasma retinol-binding protein, bile acid CoA ligase (BAL), insulin-like growth factor-I (IGF-I), ApoM, angiopoietin-like protein 3 (Angptl3) and lysosomal-associated protein transmembrane-4 beta (LAPTM4B) were increased as well in this phase. Plasma retinol-binding protein could transport retinol and vitamin A, whose increase in 8-16 h after PH showed that retinol and vitamin A were essential to hormone synthesis in LR[22]. BAL could facilitate adsorption of amino acids by catalyzing conjugation of bile acids with amino acids[23]. IGF-I was found to be involved in the liver and brain growth and development of embryos[24]. Its downregulation in 8-24 h after PH indicated that cell cycle was in the phase of DNA synthesis. The reduction of ApoM in 8-24 h after PH was supposed to promote lipolysis and to provide energy for LR. Angptl3 is a hepatic secretory factor, which could activate lipolysis in adipocytes by response to the liver X receptor[25]. LAPTM4B, a novel gene was upregulated in hepatic carcinoma, whose N-terminus is essential for the survival of cells[26]. The downregulation of LAPTM4B may be related to refraining cell necrosis in LR.

Genes expression started in the intermediate phase of LR

In the intermediate phase of LR (16-24 h after PH), hepatocytes synthesized DNA. It was confirmed that 41 genes were expressed at 12 h after PH. The increase of mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) at 12 h was assumed to provide enough ATP for DNA synthesis. NAD(P)H steroid dehydrogenase (Nsdhl), hepatic
cytochrome P450 cholesterol 7 alpha-hydroxylase (CYP7) and ATP-binding cassette were induced in this time, which may play a role in the conversion of lanosterol into cholesterol[27,28]. L28 is a component of 70S ribosome, whose upregulation in 12-24 h after PH was supposed to help 70S ribosome conformation and to accelerate protein synthesis. Activator protein 1 (AP1)-like elements and P53 were increased, which probably prevent injured liver from apoptosis and necrosis[29]. The proteins associated with protein fold such as cathepsin C (Ctsc), dipeptidyl aminopeptidase I (Ddpep1), Hsp40 and hsp70 (p73/p72) were also up expressed, which are essential to form the correct structure of proteins.

**Genes expression started in the early-late phase of LR**

In the early-late phase of LR (24-36 h after PH), hepatocytes must complete all the activations of later S phase, G2 phase and M phase. It was checked that seven genes were started to express at 24 h after PH. Five of them were upregulated and two were downregulated, of which, the restrain of bifunctional aminoacyl-tRNA synthetase was up expressed at 24 h after PH, which may play a pivotal role in separating the subunits of the eukaryotic tRNA synthetase complex in LR. Furthermore, eukaryotic release factor 3 incorporated in a complete scheme for translation termination of proteins, whose induction may be advantageous to release essential proteins to exert functions. The mRNA level of sialyltransferase was increased at 36 h, suggesting that liver releases large amounts of sialyltransferase to mediate recovery of liver functions after PH.

**Genes expression in the late phase of LR**

In the late phase of LR (24-36 h after PH), hepatocytes went through the second cell cycle, and other cells began to divide. It was found that seven genes were induced to express at 36 h after PH. Of them, coagulation factor 2 inhibitor was upregulated, which facilitates blood circulation in the regenerating liver. The pregnancy-zone protein (Pzp) was induced, which accelerates hepatocyte division and mediate cell differentiation in LR. Complement component 1 was increased in 48-72 h after PH, which protects against the outer intruders in the injured liver.

**Genes expression started in the terminal phase of LR**

In the terminal phase of LR (72-144 h after PH), structure and function of the regenerating liver were recovered. It was found that 15 genes were induced in 48-144 h after PH. Of them, galactose-specific genes such as membrane-bound C-reactive protein were increased, demonstrating that galactose is necessary in terminal phase of LR. Cathepsin D was decreased, suggesting that cell migration was suppressed[28]. CDK110 was upregulated at 72 h after PH, which mediates cell differentiation in LR. Flavanol cocoa was continuously induced in LR, which can prevent the regenerated liver from early alcohol-induced liver injury[30].

In summary, we have screened 576 genes from subtracted cDNA libraries and made an in-house cDNA microarray. These genes were highly and specifically expressed in the liver. Using the chips, we performed a large-scale analysis of gene expression in LR and found that the expressions of 133 reported and 167 unreported genes were altered more than twofolds at one or more time points. Cluster analysis showed gene expression patterns at 2 and 4 h, 12 and 16 h, 48 and 96 h as well as 72 and 144 h after PH had strong correlations respectively. The 133 reported genes could be categorized into six distinct temporal patterns of induction and were involved in 24 groups of proteins. Their actions in LR were discussed. However, to elucidate the mechanism of LR, further research is needed.

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