



Expression pattern and action analysis of genes associated with the responses to chemical stimuli during rat liver regeneration

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Abstract

AIM: To study the genes associated with the responses to chemokines, nutrients, inorganic substances, organic substances and xenobiotics after rat partial hepatectomy (PH) at transcriptional level.

METHODS: The associated genes involved in the five kinds of responses were obtained from database and literature, and the gene expression changes during liver regeneration in rats were checked by the Rat Genome 230 2.0 array.

RESULTS: It was found that 60, 10, 9, 6, 26 genes respectively participating in the above five kinds of responses were associated with liver regeneration. The numbers of initially and totally expressed genes occurring in the initial phase of liver regeneration (0.5-4 h after PH), G₀/G₁ transition (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-functional reconstruction (66-168 h after PH) were 51, 19, 52, 6 and 51, 43, 98, 68 respectively, illustrating that the associated genes were mainly triggered in the initiation and transition stages, and functioned at different phases. According to their expression similarity, these genes were classified into 5 groups: only up-regulated (47), predominantly up-regulated (18), only down-regulated (24), predominantly down-regulated (10), and up- and down-regulated (8). The total times of their up-regulated and down-regulated expression were 441 and 221, demonstrating that the number of up-regulated genes is more than that of the down-regulated genes. Their time relevance and gene expression patterns were classified into 14 and 26 groups, showing that the cell physiological and biochemical activities

were staggered, diversified and complicated during liver regeneration in rats.

CONCLUSION: The chemotaxis was enhanced mainly in the forepart and metaphase of LR. The response of regenerating liver to nutrients and chemical substances was increased, whereas that to xenobiotics was not strong. One hundred and seven genes associated with LR play important roles in the responses to chemical substances.

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Key words: Partial hepatectomy; Rat Genome 230 2.0 array; Responses to chemical substances; Genes associated with liver regeneration

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INTRODUCTION

The liver has tremendous capacity to regenerate itself^[1,2]. Liver cells proliferate rapidly to compensate the lost liver tissues after liver injury or chemical stimulus, which is called liver regeneration (LR)^[1,3]. Generally, based on the cell physiological activities, the regeneration process is classified into 4 phases: the initiation (0.5-4 h after hepatectomy, PH), the transition from G₀ to G₁ (4-6 h after PH), the cell proliferation (6-66 h after PH), the cell differentiation and structure-functional reorganization (66-168 h after PH)^[2]. According to the time course, it is divided into 4 phases: forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH), and anaphase (72-168 h after PH)^[4], involving hepatic cell activity, dedifferentiation, proliferation and its regulation, redifferentiation, structure-functional reorganization^[5], which are regulated by many factors including chemical substances^[6].

Liver is a vital organ where chemical substances undergo transformation^[7-9], it also can respond rapidly to chemical stimulus^[10]. This process involves more than 400

genes, so high-throughput biological analysis is needed^[11,12]. The pathogenesis of drug-induced liver disease, toxic liver injury and the characteristics of responses of regenerating liver to drug, toxin, oxidation and unfolded protein have been studied at transcriptional level^[13]. In this paper, the expression changes of the genes in rat regenerating liver after PH were detected by Rat Genome 230 2.0 array^[14]. Two hundred genes were related to the response to chemical substances, in which, 107 genes were observed to be associated with liver regeneration^[11]. Expression changes, patterns and action of the genes were primarily analyzed.

MATERIALS AND METHODS

Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were separated into groups randomly, each group consisting of 6 rats (male: female = 1:1). PH was performed according to Higgins and Anderson^[15], the left and middle lobes of liver were removed. Rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time point. The livers were rinsed three times in PBS at 4°C, then 100-200 mg livers from middle parts of right lobe (total $0.1-0.2 \times 6$ g liver tissues, six samples for each group) were collected and stored at -80°C. The sham-operation (SO) groups were the same with PH groups except that the liver lobes were not removed. The laws of animal protection of China were enforced strictly.

RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of Trizol kit (Invitrogen)^[16] and then purified based on the instructions of RNeasy mini-kit (Qiagen)^[17]. Total RNA samples were found to exhibit a 2:1 ratio of 28S to 18S rRNA intensities by agarose electrophoresis (180 V, 0.5 h). Total RNA concentration and purity were estimated by optical density measurement at 260/280 nm^[18].

cDNA, cRNA synthesis and purification

As template, 1-8 µg total RNA was used for cDNA synthesis. cDNA and cRNA synthesis was carried out based on method of Affymetrix^[19]. cRNA labeled with biotin was synthesized using 12 µL of the above synthesized cDNA as the template, and cDNA and cRNA were purified according to the sample purification procedure of GeneChip Analysis^[19]. Measurement of the concentration, purity and quality of cDNA and cRNA were the same as above^[18].

cRNA fragmentation and microarray detection

Fifteen µL (1 µg/µL) cRNA incubated with $5 \times$ fragmentation buffer at 94°C for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarray produced by Affymetrix, then hybridization was carried out for 16 h at 45°C on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics

station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip Scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed^[14].

Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (R_i) were obtained by quantifying and normalizing the signal values using GCOS1.2^[14].

Normalization of microarray data

To minimize error in the microarray analysis, each analysis was performed three times by Rat Genome 230 2.0 microarray. The results in which the total ratio(R^m) was maximal and the average of the three housekeeping genes β-actin, hexokinase and glyceraldehyde-3-phosphate dehydrogenase approached 1.0 (R^h), were taken as a reference. The modified data were generated by applying a correction factor (R^m/R^h) multiplying the ratio of every gene in R^h at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by NAP software (normalization analysis program) according to the cell cycle progression of the regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel software^[14,20,21].

Identification of genes associated with liver regeneration

The nomenclature of the responses to chemical substances was adopted from the GENEONTOLOGY database (www.geneontology.org), and input into the database at NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to identify the rat, mouse and human genes associated with the responses to chemical substances. According to KEGG (www.genome.jp/kegg/pathway.html#amino) and BIOCARTA (www.biocarta.com/genes/index.asp), the genes associated with the biological process were collated. The results of this analysis were codified, and compared with the results obtained for mouse and human searches in order to identify human and mouse genes which are different from those of rat. Comparing these genes with the analysis output of the Rat Genome 230 2.0 array, those genes which showed a greater than twofold changes in expression level, and observed as meaningful expression changes^[22], were referred to as rat homologous or rat specific genes associated with the responses to chemical substances under evaluation. Genes, displaying reproducible results with three independent analyses with the chip and showing a more than twofold change in expression level in at least one time point during liver regeneration with significant difference ($0.01 \leq P < 0.05$) or extremely significant difference ($P \leq 0.01$) between PH and SO, were considered as associated with liver regeneration.

RESULTS

Expression changes of genes associated with responses to chemical substances during liver regeneration

According to the data from databases at NCBI, GENEMAP, KEGG, BIOCARTA and RGD, 403 genes

Table 1 Expression abundance of 107 genes associated with responses to chemical substances during rat liver regeneration

Gene Abbr.	Associated to others	Fold difference	Gene Abbr.	Associated to others	Fold difference	Gene Abbr.	Associated to others	Fold difference	Gene Abbr.	Associated to others	Fold difference
1 Chemotaxis			Fosl1		2.3	Utrn		2.4	Ppp2cb		4.5
C3ar1		0.3, 2.3	G1p2		22.6	Vegfa		0.1, 4.5	Rela		0.5
C5r1		0.4, 2.6	Gpr2		0.4, 4	Xcl1		0.1, 3.7	5 Response to xenobiotic		
Ccl17		0.1	Htr6		9.2	2 Response to nutrient			Abhd6		0.1
Ccl19		3.9	Ifng		6.5	Cckar		0.3, 3.2	Afp		0.1, 3.7
Ccl2		128	Il16		0.4, 3.1	Enpp1		2.2	Ahr		2.2
Ccl20		8	Il1b		0.4	Gipr		0.3, 2.3	Ahrr		0.1
Ccl21B		0.1, 2	Il4		0.1, 2.6	Gnai2		2.3	Akr1c12		0.2, 2.2
Ccl24		4	¹ Itga1		2.9	¹ Kpna2		4.3	Apoa2		2.9
Ccl27		0.3	Itgam		3.4	Ogt		2.7	Arnt2		0.4, 6.8
Ccl4		0.2, 3	Itgb2		0.5	Sst		0.2, 4.3	Cda		0.1
Ccl7		22.6	Kit		0.4	Sstr2		0.4, 4.9	Chga		2.3
Ccr1		0.4, 27.9	Lsp1		0.3	Stc1		2.3	Cyp1a1		59.7
Ccr6		0.3, 4.3	Map2k1		9.1	Stc2		4	Cyp2c		52.6
Ccr11		3.7	Mapk1		2.7	3 Response to inorganic substance			Cyp3a13		0.2
Cklf1		8.3	Plau		0.4, 3				Defb3		0
Cklfsf1		0.3, 2.1	Plaur		13.9	Als2		0.5	Ephx1		0.4, 2.8
Cklfsf3		4.2	Pld1		2.3	¹ Aqp9	4	0.4, 3.7	Gstm2		4.5
Cmklr1		2.4	Plp2		0.4, 4.3	Arsb		2	Gstm4		7.5
Cmkor1		7.1	Ppbbp		0.1, 2.1	Arse		0.2, 2.5	Laptm4a		2.5
Creb3		8.4	¹ Prkca		4.6	As3mt	5	0.5	Lpo		3.4
Cx3cr1		0.4, 6.8	Ptafr		7.1	Asna1		0.5	Mbp		0.4
Cxcl10		0.3, 9.2	Rac2		0.5	Atp7a		0.4	Pap		68.6
Cxcl12		0.2	Rala		2.3	Drg1	5	0.4	Prg2		0.2
Cxcl16		0.3	Scg3		0.5, 2.2	Zd10b		5.6	Ugt1a5		2.1
Defb1	5	0.4, 2.5	Serpind1		0.1	4 Response to organic substance			Wbscr21		0.5
Ela2		0.5, 52	Spn		0.2, 4	Accn1		0.3, 10.1			
Enpp2		6.2	Spp1		0.5, 2.7	Accn3		0.5, 4			
Fgf2		0.5, 2.1	Tgfb2		0.5, 2.9	Ppp2ca		3			

¹Reported genes associated with liver regeneration; Associated to others: involved in the other kind of responses to chemical substances.

were involved in the responses to chemical substances, 200 of them were contained in the Rat Genome 230 2.0 array. Among the 200 genes, 107 displayed meaningful changes at least at one time point after PH, there was significant difference or extremely significant difference in expression between PH and SO groups, and results were reproducible from the three checks with Rat Genome 230 2.0 array, suggesting that the genes were associated with liver regeneration. The up-regulation was 2-128 folds, and the down-regulation was 2-10 folds that of controls (Table 1). The analysis indicated that 47 genes were up-regulated, 24 down-regulated, and 36 up/down-regulated during liver regeneration. The total up- and down-regulated times were 441 and 221, respectively (Figure 1A). At the initial phase, 34 genes were up-regulated and 16 down-regulated; at the transition phase from G₀ to G₁, 24 genes were up-regulated and 14 down-; at the cell proliferation phase, 47 genes were up-, 26 down- and 24 up/down-regulated; at cell differentiation and structure-function reorganization phase (66-168 h after PH), 44 genes were up-, 17 down- and 6 up/down-regulated (Figure 1B).

Initial expression time of genes associated with responses to chemical substances during liver regeneration

At each time point of liver regeneration, the numbers of initially up-, down-regulated and totally up-, down-regulated genes were in the following sequence: both 10 and 3 at 0.5 h; 11, 6 and 21, 8 at 1 h; 6, 1 and 18, 4 at 2 h; 8, 6 and 25, 8 at 4 h; 3, 2 and 19, 11 at 6 h; 1, 1 and 14, 9 at 8 h; 2, 3 and 20, 11 at 12 h; 10, 4 and 28, 7 at 16 h; 2, 10 and 26, 22 at 18 h; 2, 0 and 24, 12 at 24 h; 5, 1 and 19, 7 at 30 h; 0, 0 and 20, 15 at 36 h; 1, 0 and 17, 4 at 42 h; 0, 0 and 26, 18 at 48 h; 1, 1 and 22, 18 at 54 h; 1, 0 and 24, 11 at 60 h; 2, 0 and 22, 9 at 66 h; 1, 0 and 19, 10 at 72 h; 2, 0 and 25, 10 at 96 h; 0, 1 and 14, 10 at 120 h; 0, 0 and 15, 7 at 144 h; 0, 0 and 13, 7 at 168 h (Figure 2). Generally, gene expression changes occurred during the whole liver regeneration, and the up- and down-regulated times were 441 and 221, respectively. The initially up-regulated genes were predominantly expressed in the forepart, prophase and pre-metaphase, and only few genes were initially expressed in the metaphase, pre-anaphase and mid-anaphase, whereas no initial expression in the late anaphase.

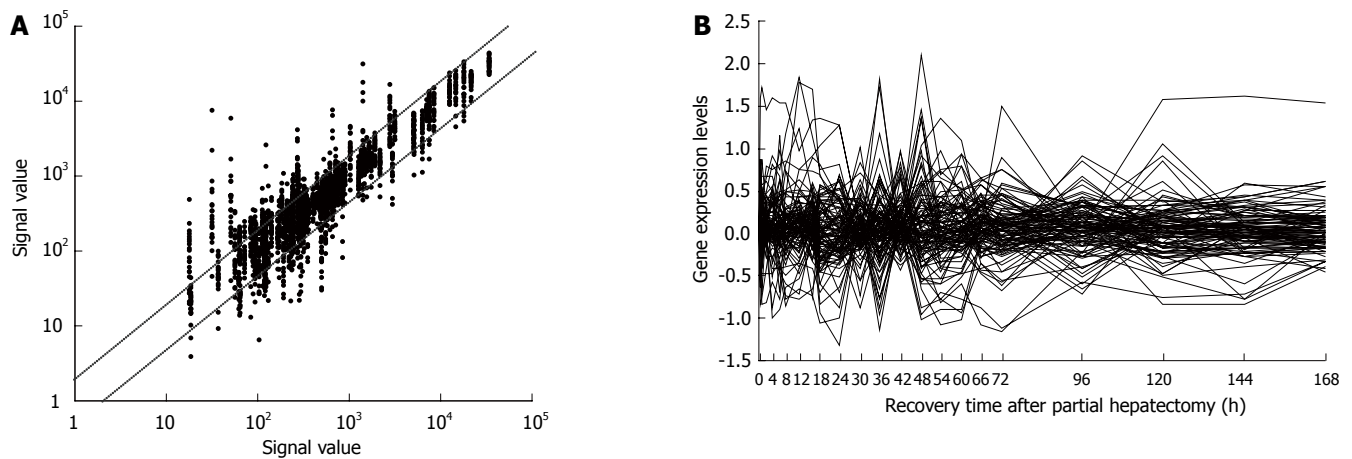


Figure 1 Expression frequency, abundance and changes of 107 genes associated with responses to chemical substances during rat liver regeneration. Detection data of Rat Genome 230 2.0 array were analyzed and graphed by Microsoft Excel. **A:** Gene expression frequency. The dots above bias represent the genes up-regulated more than 2-fold, those under bias represent the ones down- more than 2-fold, and total times of up- and down-regulation were 441 and 221; the ones between two diagonal lines represent the genes without alteration in expression; **B:** Gene expression abundance and changes. Eighty-three genes were 2-128 folds up-regulated, and sixty genes 2-10 folds down-regulated.

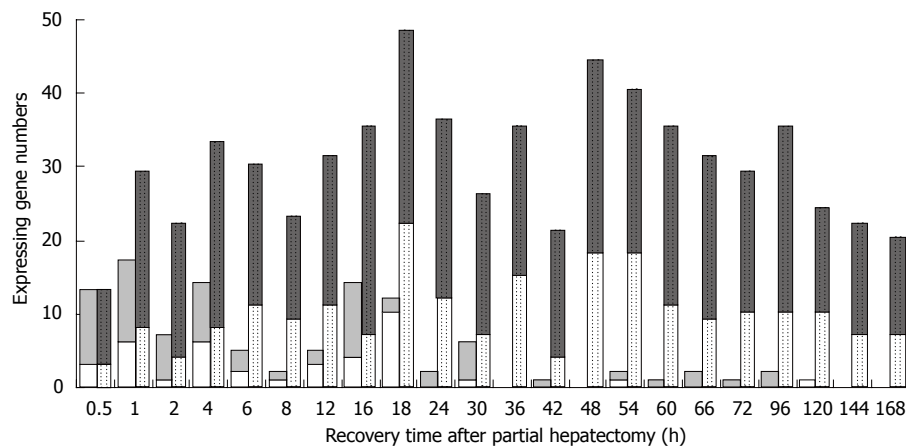


Figure 2 The initial and total expression profiles of 107 genes associated with responses to chemical substances at each time point of liver regeneration. Grey bars: up-regulated genes; White bars: down-regulated genes. Blank bars represent initially expressing genes, in which up-regulation genes are predominantly in the forefront, prophase and early metaphase, and few genes initially expressed in the metaphase, early anaphase and middle anaphase, whereas no initial expression in the late anaphase. Dotted bars represent the total expressing genes, in which some genes are up-expressed, and the others down-expressed during LR.

Expression similarity and time relevance of the genes associated with the responses to chemical substances during liver regeneration

Among the 107 genes characterized, 47 were up-regulated, 18 predominantly up-, 24 only down-, 10 predominantly down-, 8 up/down-, based on their similarity in expression (Figure 3). According to the time relevance, they were classified into 14 groups: 0.5 and 1 h, 2 and 4 h, 6 h, 8 and 12 h, 16 h, 18 and 24 h, 30 and 42 h, 36 h, 48 h, 54 and 60 h, 66 h, 72 and 120 h, 96 h, 144 and 168 h. The up- and down-regulated times were 31 and 11, 43 and 12, 19 and 11, 34 and 20, 28 and 7, 50 and 34, 36 and 11, 20 and 15, 26 and 18, 46 and 29, 22 and 9, 36 and 20, 25 and 10, 28 and 14 (Figure 3). The up-expressed genes were mainly associated with chemotaxis, xenobiotics metabolism, protein transport and apoptosis development, and the down-expressed genes were mostly the ones associated with transportation and mucosal defense.

Expression patterns of genes associated with responses to chemical substances during liver regeneration

The expressions of 107 genes were categorized into 26 patterns according to the expression trends. Figure

4A-4G shows the up-regulated trends of gene expression profiles after PH, involving 14 patterns (47 genes); Figure 4H-4K shows the opposite expression trends, involving 9 patterns (24 genes); Figure 4L,4M,4N respectively displays that 18 genes were predominantly up-regulated, 10 genes predominantly down-regulated and 8 genes similarly up/down-regulated, involving 3 patterns.

DISCUSSION

Liver is an important site of substance transformation, and it can also rapidly respond to chemical stimuli. During the process, the proteins associated with chemotaxis are as following: nine receptors bind with corresponding chemokine ligand to activate chemotaxis^[23]; eighteen chemokine factors and chemokine-like factors, such as chemokine CC ligand 2 (CCL2), bind to corresponding chemokine receptors to activate chemotaxis^[24]; eleven proteins including protein kinase C alpha (PRKCA) participate in chemotaxis^[25,26]; five proteins such as plasminogen activator urokinase receptor (PLAUR), accelerate cell migration^[27]; three proteins including integrin alpha 1 (ITGA1), contribute to cell adhesion^[28].

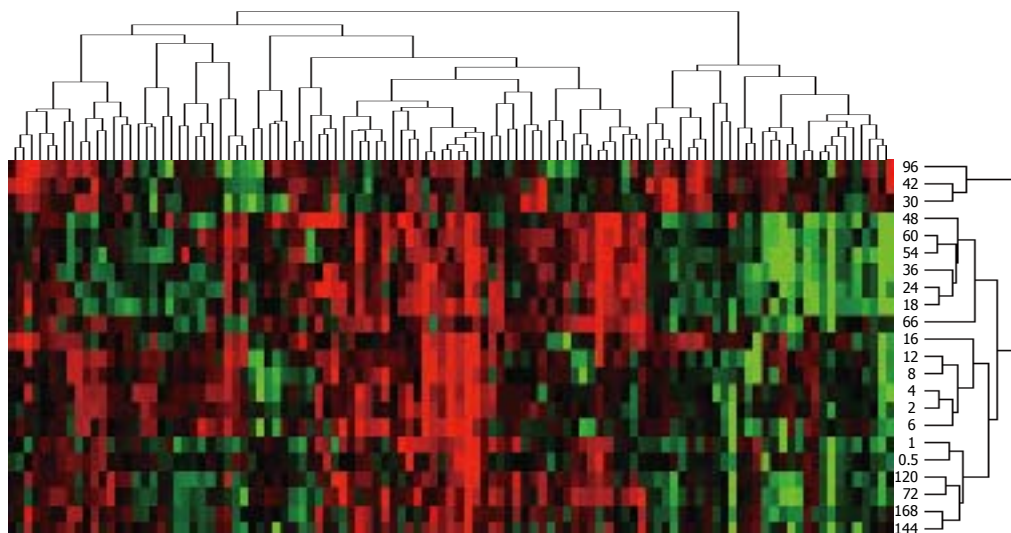


Figure 3 Expression similarity and time relevance clusters of 107 genes associated with responses to chemical substances during LR. Detection data of Rat Genome 230 2.0 array were analyzed by H-clustering. Red represents up-regulated genes mainly associated with chemotaxis, xenobiotics metabolism, protein transport and apoptosis development; Green represents down-ones mainly associated with transportation and mucosal defense; Black: the genes having insignificant expression changes. The upper and right trees respectively show expression similarity and time series clusters, by which the above genes were classified into 5 and 14 groups.

The genes encoding the above proteins showed similar expression profiles at some points while different at other points, indicating that they may co-regulate chemotaxis. Among them, *prkca* was up-regulated at 16, 30, 42 and 96 h after PH, and reached a peak at 96 h that is 4.6 times higher than the control; *itga1* was up-regulated at 6-24 and 54 h, reaching a peak at 8 h, 2.9 times higher than the control. The two genes are generally consistent with the result reported by Dransfeld *et al.*^[11]. *acr1* was up-regulated at 8-36, 48 and 120 h, and peaked at 48 h, 27.9 times that of control. *cc2* was up-regulated at 0.5-1, 12-24, 36, 48-72 and 96 h, and reached a peak at 48 h, 128 times that control. *plaur* was up-regulated at 1, 6, 18-24, 48, 72 and 120 h, and peaked at 6 h, 13.9 times higher than the control, presuming that they play a key role in inflammation during liver regeneration.

Karyopherin alpha 2 (KPNA2) involved in glucose response, transports karyophilic proteins into the nucleus^[29,30]. Cholecystokinin A receptor (CCKAR) promotes pancreatic enzyme secretion and smooth muscle contraction of stomach and gallbladder^[31]. Three proteins including somatostatin (SST) inhibit gastrointestinal hormone release^[32]. The meaningful expression profiles of the genes encoding the above-mentioned proteins show the identity or similarity at some points, whereas difference at others, presuming that they may co-regulate absorption and consumption of nutrients. Especially, *kpna2* displayed up-regulation at 16-24, 36 and 48-96 h, and had the highest abundance at 60 h, 4.3 times that of control. This result was basically in line with Dransfelds' result^[11]. *ckar* appeared at 0.5-24, 36 and 48-54 h, and had peak expression at 8 h, 46.2 times that of control. *sst* was up-regulated at 4-24, 36, 48-60 and 120 h, and peaked at 12 h, 4.3 times that of control, presuming that they play a crucial role in nutrients assimilation and utilization in regenerating liver.

Aquaporin 9 (AQP9) influenced by inorganic substances, which could be inactivated by Hg^{2+} , stimulates transmembrane transportation of water and a variety of noncharged solutes, including urea^[33]. It was up-regulated at 0.5-1, 24 and 54-72 h, and reached a peak at 66 h, being 3.7 times higher than the control, but the result was not

consistent with Dransfelds' at 6 h^[11], and need to be further analyzed with Northern blotting. Zinc responsive protein beta (ZD10B) stimulated by Zn^{+} plays a role in apoptosis^[34], and was up-regulated at 16-24, 36, 48-72 and 144-168 h, and reached a peak at 72 h, 5.6 times higher than control, indicating that the two play crucial roles in inorganic substances transport and removal in regenerating liver.

Amiloride-sensitive cation channel 1 (ACCN1) affected by organic substances, plays a part in neurotransmission and Na^{+} transport^[35], and is up-regulated at 30 and 42 h with the highest abundance of 10 folds at 30 h. Protein phosphatase 2a catalytic subunit beta (PPP2CB) stimulated by ceramide is implicated in the negative control of cell growth and division by dephosphorylation^[36,37]. It was up-regulated at 16, 30, 42 and 120 h, and had abundance peak at 42 h, 4.5 folds of control, presuming that the two genes play a crucial part in response to organic substances in regenerating liver.

In response to xenobiotics, fourteen enzymes including cytochrome P450 family 1 subfamily a 1 (CYP1A1), metabolize xenobiotics^[38]. Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) enhances expression of oxygen-responsive genes^[39]. Alpha-fetoprotein (AFP) is involved in transportation of copper and nickel^[40]. The meaningful expression profiles of the genes encoding the above proteins are the same or similar at some points while different at others, indicating that they may co-regulate the response to xenobiotics. Notably, *cyp1a1* was up-regulated at 0.5-24, 36, 48-72 and 120 h, and reached a peak at 12 h, 59.7 times higher than the control. *arnt2* was up-regulated at 30-42, 60, 72 and 120 h, and reached a peak at 30 h, 6.8 times higher than the control. *afp* was up-regulated at 1 and 54-72 h, and reached a peak at 66 h, 3.7 times higher than the control, showing that they play a critical role in xenobiotics biotransformation in regenerating liver.

In conclusion, the expression changes of the genes associated with chemotaxis, the responses to nutrients, inorganic substances, organic substances and xenobiotics during liver regeneration in rats were investigated by high-throughput gene expression analysis. It was primarily proved that chemotaxis and the responses to the chemical substances are enhanced during LR, that Rat Genome 230

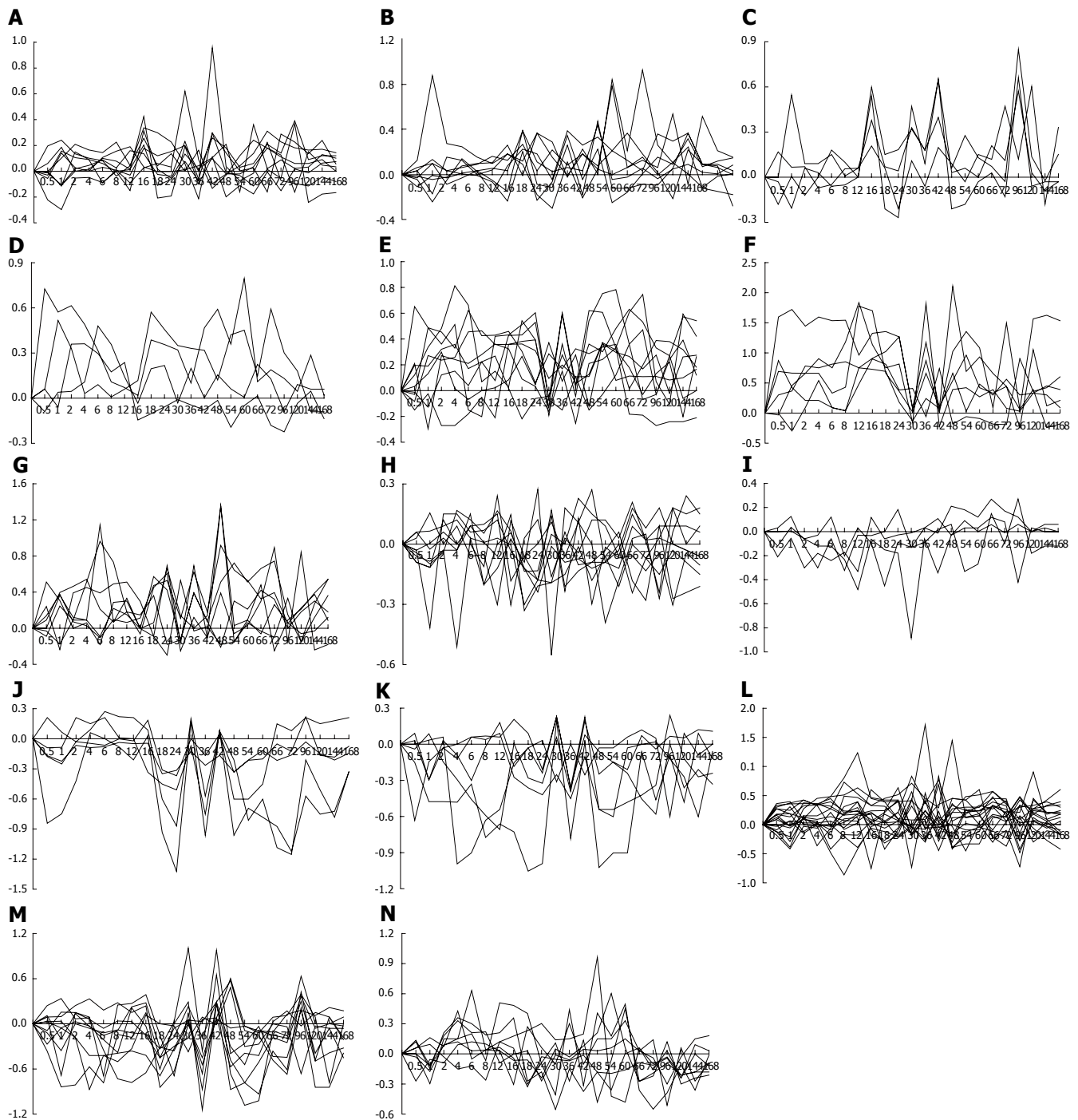


Figure 4 Gene expression patterns of 107 genes associated with responses to chemical substances during LR. Twenty-six expression patterns were obtained by analysis of data detection of Rat Genome 230 2.0 array with Microsoft Excel. **A-G**: 47 up-regulated genes; **H-K**: 24 down-regulated genes; **L-N**: 36 up/down-regulated genes. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of signal values of genes at each time point vs control.

2.0 array is a useful tool analyzing the above responses at the transcriptional level. However, the process DNA \rightarrow mRNA \rightarrow protein was influenced by many factors including protein interaction. Therefore, the above results need to be further analyzed by the techniques, such as Northern blotting, protein chip, RNA interference, protein-interaction, etc.

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