



RAPID COMMUNICATION

## Expression patterns and action analysis of genes associated with physiological responses during rat liver regeneration: Innate immune response

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Supported by the National Natural Science Foundation of China, No. 30270673

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Received: 2006-08-25 Accepted: 2006-10-09

### Abstract

**AIM:** To study the relationship between innate immune response and liver regeneration (LR) at transcriptional level.

**METHODS:** Genes associated with innate immunity response were obtained by collecting the data from databases and retrieving articles. Gene expression changes in rat regenerating liver were detected by rat genome 230 2.0 array.

**RESULTS:** A total of 85 genes were found to be associated with LR. The initially and totally expressed number of genes at the phases of initiation [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-function reconstruction (66-168 h after PH) was 36, 9, 47, 4 and 36, 26, 78, 50, respectively, illustrating that the associated genes were mainly triggered at the initial phase of LR and worked at different phases. According to their expression similarity, these genes were classified into 5 types: 41 up-regulated, 4 predominantly up-regulated, 26 down-regulated, 6 predominantly down-regulated, and 8 approximately up/down-regulated genes, respectively. The expression of these genes was up-regulated 350 times and down-regulated 129 times respectively, demonstrating that the expression of most genes was enhanced while the expression of a small number of genes was decreased during LR. Their time relevance was classified into 14 groups, showing that the cellular physiological and biochemical activities during LR were staggered. According to the gene expression patterns,

they were classified into 28 types, indicating that the cellular physiological and biochemical activities were diverse and complicated during LR.

**CONCLUSION:** Congenital cellular immunity is enhanced mainly in the forepart, prophase and anaphase of LR while congenital molecular immunity is increased dominantly in the forepart and anaphase of LR. A total of 85 genes associated with LR play an important role in innate immunity.

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**Key words:** Partial hepatectomy; Rat genome 230 2.0 array; Innate immune response; Genes associated with liver regeneration

Chen GW, Zhang MZ, Zhao LF, Xu CS. Expression patterns and action analysis of genes associated with physiological responses during rat liver regeneration: Innate immune response. *World J Gastroenterol* 2006; 12(48): 7852-7858

<http://www.wjgnet.com/1007-9327/12/7852.asp>

### INTRODUCTION

Organisms can resist and remove endogenous and exogenous poisons *via* their innate immune cells and other factors. This process is known as innate immune response<sup>[1]</sup>, a self-protection mechanism of living organisms which is absolutely indispensable to their survival<sup>[2]</sup>. Innate immune responses consist of three parts, namely barrier of self-tissue, innate cellular immunity and innate molecular immunity. Tissue barrier can excrete antibacterial and bactericidal matters to kill pathogens, innate cellular immunity can not only remove pathogens invading body *via* immune cells but also clear the broken, dead and abnormal cells, while innate molecular immunity can demolish and dissolve injurious substances *via* active molecules and cytokines<sup>[3]</sup>. Liver containing NK cells, T lymphocytes, macrophages, *etc*, is an important organ where innate immune response takes place<sup>[4]</sup>. After partial hepatectomy (PH), liver undergoes severe injury. How the remnant liver cells are protected by the innate immune system deserves intensive study<sup>[5]</sup>.

In addition, PH<sup>[6]</sup> can activate the remaining hepatocytes to rapidly proliferate and compensate for the

loss of liver mass, which is known as liver regeneration (LR)<sup>[7,8]</sup>. Based on the cellular physiological activities, the regeneration proceeding is usually categorized into four stages: initiation (0.5-4 h after PH), transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and reorganization of the structure-function (66-168 h after PH)<sup>[8]</sup>. According to the time course, it can be classified into four 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)<sup>[9]</sup>, involving many physiological and biochemical events, such as cell activation, cell de-differentiation, cell proliferation and its regulation, cell re-differentiation, reorganization of structure-function<sup>[10]</sup>, which are regulated by many factors including innate immune response. The action of genes associated with liver diseases caused by hepatitis virus infection and pathogen infection during LR, has been analyzed<sup>[11-13]</sup>. In the present study, rat genome 230 2.0 array containing 151 genes involved in innate immune response was used to detect the gene expression changes in regenerating liver after 2/3 hepatectomy as previously described<sup>[14-17]</sup> in order to investigate the relevance between LR and innate immune response at transcriptional level. The expression changes, patterns and action of these genes during LR were primarily analyzed. Our results indicate that 85 out of the 151 genes are associated with LR<sup>[18]</sup>.

## 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 divided into groups at random, 6 rats in each group (male: female = 1:1). PH was performed as previously described<sup>[6]</sup>, the left and middle lobes of liver were removed. The 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 points. The livers were rinsed three times in PBS at 4°C, then 100-200 mg liver tissue was taken from middle part of the right lobe. Six samples were collected from each group and mixed into 1-2 g (0.1-0.2 g × 6) liver tissue, then stored at -80°C. The sham-operation (SO) groups underwent the same PH without removal of the liver lobes. The animal protection laws of China were strictly followed.

### *RNA isolation and purification*

Total RNA was isolated from frozen livers according to the manual of Trizol kit (Invitrogen)<sup>[19]</sup> and then purified based on the guide of RNeasy mini kit (Qiagen)<sup>[20]</sup>. Agarose electrophoresis (180V, 0.5h) showed that total RNA sample exhibited a 2:1 ratio of 28S to 18S rRNA intensities. Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm<sup>[21]</sup>.

### *cDNA, cRNA synthesis and purification*

Total RNA (1-8 µg) was used as a template for cDNA

synthesis. cDNA and cRNA synthesis was proceeded as previously described<sup>[16]</sup>. cRNA labeled with biotin was synthesized using 12 µL synthesized cDNA as a template, cDNA and cRNA were purified<sup>[16]</sup>. Measurement of concentration, purity and quality of cDNA and cRNA was performed as previously reported<sup>[21]</sup>.

### *cRNA fragmentation and microarray detection*

Fifteen µL (1 µg/µL) cRNA incubated with 5 × 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, and 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<sup>[17]</sup>.

### *Microarray data analysis*

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2<sup>[17]</sup>.

### *Normalisation of microarray data*

To minimize the errors in microarray analysis, each analysis was performed three times by rat genome 230 2.0 microarray. Results with a maximal total ratio ( $R^m$ ) and an average of three housekeeping genes  $\beta$ -actin, hexokinase and glyceraldehyde-3-phosphate dehydrogenase approaching 1.0 ( $R^h$ ) were taken as a reference. 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 normalization analysis program (NAP) software according to the cell cycle progression of regenerating hepatocytes. Data statistics and cluster analysis were done using the GeneMath, GeneSpring, Microsoft Excel softwares<sup>[17,22,23]</sup>.

### *Identification of genes associated with liver regeneration*

First, the nomenclature of innate immune response was adopted from the GENEONTOLOGY database ([www.geneontology.org](http://www.geneontology.org)) and input into NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and RGD ([rgd.mcw.edu](http://rgd.mcw.edu)) to identify the rat, mouse and human genes associated with the biological process. According to the maps of biological pathways embodied by GENMAPP ([www.genmapp.org](http://www.genmapp.org)), KEGG ([www.genome.jp/kegg/pathway.html#amino](http://www.genome.jp/kegg/pathway.html#amino)) and BIOCARTA ([www.biocarta.com/genes/index.asp](http://www.biocarta.com/genes/index.asp)), genes associated with innate immune response were collated. The results of this analysis were codified and compared with those obtained for humans and mice in order to identify human and mouse genes which are different from those of rats. Comparing these genes with the analysis output of rat genome 230 2.0 array, genes showing more than twofold change in expression level as meaningful expression changes<sup>[18]</sup>, were referred to as rat homologous or rat specific genes associated with innate immune response

**Table 1** Expression of 85 innate immune response-associated genes 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
Innate immune cells				Colec12			3.9	Bdkrb2	3		0.4	Cxcl10			0.3, 9.2
1 Macrophage				Crp			0.5	<sup>1</sup> Il1b	1		0.4	Cxcl12			0.2
Adora2a		0.5		Hrh1	1		0.5, 9.9	Il1f5	1		0.4, 2.8	Darc			0.4, 8.5
Anxa1		4.3		Hrh4	1		7.5	Il1r1	1		0.5	Others			
Cebpb		3.1		Mcpt6	1, 2		0.2	Il1rn	1		16.3	Parp4			0.5
Clec7a		0.2		Nr3c1			4.7	Il2			0.3, 3.5	Alox5			0.2, 2.5
Cybb		2.5		Spp1			0.5, 2.7	Il5			3.5	Alox5ap			4.9
Ereg	2	0.4		Nfatc4			0.5	Sarm1			0.2, 4.3	Apoe			0.1
Ltb4r		0.5, 8.7		Innate immune effectors				Sele	3		12.9	Casp12			0.4, 2.6
Mif		3.2		4 Complement system				b Interferon and related factors				Dmbt1			9.8
Pap		68.6		C1qa			0.3	Ifnk			0.1, 5.7	Hck			0.4
Pla2g4a		2		C1qr1	1, 2		5.5	Ddx58			11.8	Map2k3			0.4
Ptgs2		0.1, 2.1		C2			2.1	Irf3			2.6	Prkca			4.6
S100a8		6.5		<sup>1</sup> C3			0.2	Mx2			9.4	Ptafr			7.1
S100a9		4.9		C3ar1			0.3, 2.3	c Iumor necrosis factor and related factors				Reg3a			0.1, 64.1
Tgfb1	2, 3	4.0		C4a			0.5	Ager			0.4	Reg3g			0.3, 7.5
2 NK cell				C4bpa			2.0	Myd88			2.1				
Ncr3		0.3		C5ar1			0.4, 2.6	Tnf	1		3.2				
Ptger3		0.2		Cfh			2.5	d Chemotactic numerator and telated factors							
Rela		0.5		Cfi			6.4	Ccl17			0.1				
Ripk2		0.4		Cr2	3		6.0	Ccl19			3.9				
Tlr2		10.6		Crry			2.4	Ccl2			128.0				
Tlr4		0.5		Masp1			3.0	Ccl20			8.0				
3 Other cells				Mbl2			0.2	Ccl24			4.0				
Aoc3	1	6.1		5 Cell factors				<sup>1</sup> Ccl4			0.2, 3.0				
Atrn	1	4.4		a Interlenkin and related factors				Ccl7			22.6				
Clu	1, 2	3.0		Bcl3	1		0.4	Ccr1			0.4, 24.9				

<sup>1</sup>Reported genes associated with liver regeneration; associated to others: genes are involved in another kind of the responses besides one kind of innate immune responses; others: other genes associated with innate immune response, but cannot be clearly categorized.

under evaluation. Genes displaying reproducible results in three independent analyses with the chip and more than twofold change in expression level at least at one time point during LR with a significant difference ( $P \leq 0.01 < 0.05$ ) or an extremely significant difference ( $P \leq 0.01$ ) between PH and SO, were referred to as genes associated with liver regeneration.

## RESULTS

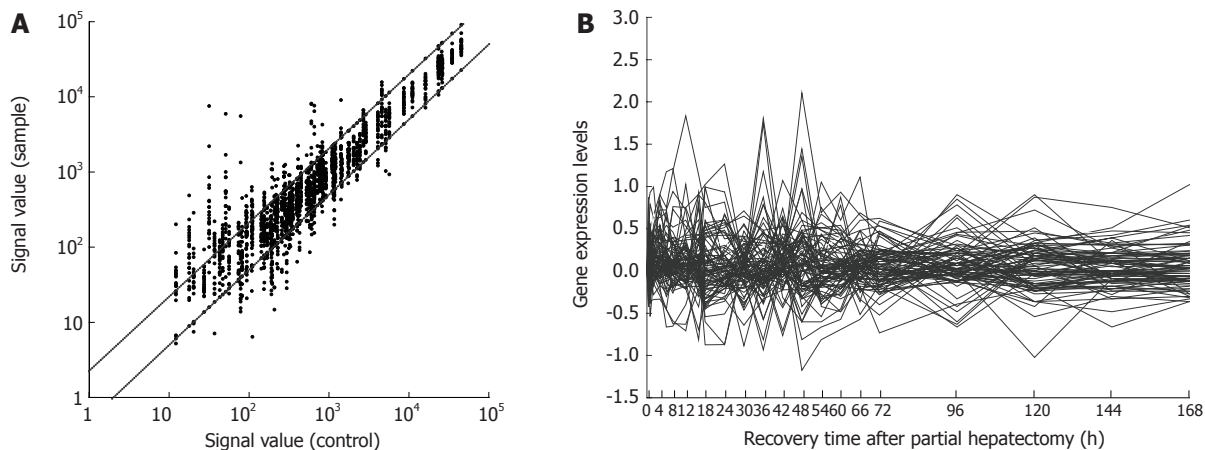
### Expression changes of innate immune response-associated genes during liver regeneration

According to the data from databases at NCBI, GENEMAP, KEGG and BIOCARTA, a total of 275 genes are involved in innate immune response, of which 151 are contained in the rat genome 230 2.0 array. In the resented study, 85 out of these 151 genes revealed meaningful expression changes at least at one time point after PH, showing a significant difference or an extremely significant difference in expression when PH was compared with SO and reproducible results checked by three detects with rat genome 230 2.0 array, suggesting that the genes were associated with LR. Their expression was up-regulated 2 to 128 times and down-regulated 2 to 10 times of the control (Table 1). The expression was up-regulated in 41, down-regulated in 26, and up/down-regulated in 18 genes, respectively, during LR. The expression of these

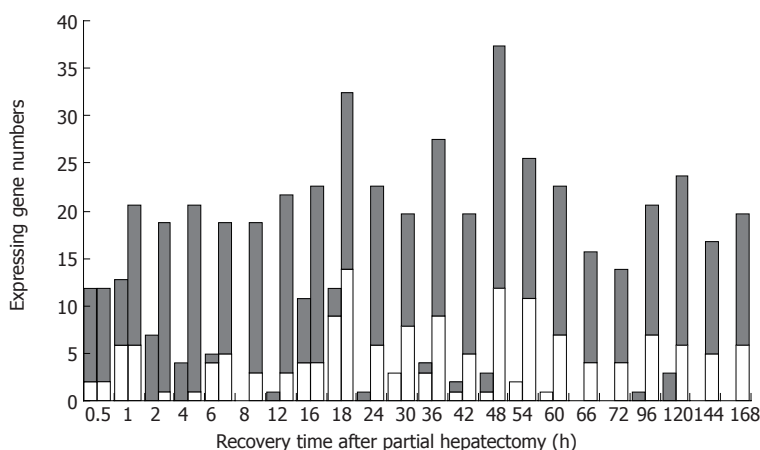
85 genes was up-regulated 350 times and down-regulated 129 times (Figure 1A). At the initiation stage of LR (0.5-4 h after PH), the expression was up-regulated in 28 and down-regulated in 8 genes. At the transition phase from G0 to G1 (4-6 h after PH), 21 was up-regulated in 21 and down-regulated in 5 genes. At cell proliferation period (6-66 h after PH), the expression was up-regulated in 42, down-regulated in 29, and up/down-regulated in 8 genes, respectively. At cell differentiation and reorganization of the structure-function stage (66-168 h after PH), the expression was up-regulated in 32, down-regulated in 14, and up/down-regulated in 4 genes, respectively (Figure 1B).

### Initiation expression time of innate immune response-associated genes during liver regeneration

At each time point of LR, the number of initially and totally up-regulated, down-regulated genes respectively was both 10 and 2 at 0.5 h; 7, 6 and 15, 6 at 1 h; 7, 0 and 18, 1 at 2 h; 4, 0 and 20, 1 at 4 h; 1, 4 and 14, 5 at 6 h; 0, 0 and 16, 3 at 8 h; 1, 0 and 19, 3 at 12 h; 7, 4 and 19, 4 at 16 h; 3, 9 and 19, 14 at 18 h; 1, 0 and 17, 14 at 24 h; 0, 3 and 12, 8 at 30 h; 1, 3 and 19, 9 at 36 h; 1, 1 and 15, 5 at 42 h; 2, 1 and 26, 12 at 48 h; 0, 2 and 15, 11 at 54 h; 0, 1 and 16, 7 at 60 h; 0, 0 and 12, 4 at 66 h; 0, 0 and 10, 4 at 72 h; 1, 0 and 14, 7 at 96 h; 3, 0 and 18, 6 at 120 h; 0, 0 and 12, 5 at 144 h; 0, 0 and 14, 6 at 166 h (Figure 2). Generally, gene expression changes occurred during the



**Figure 1** Expression frequency (A) and changes (B) of 85 innate immune response-associated genes during rat liver regeneration. Data detected by rat genome 230 2.0 array were analyzed and graphed by Microsoft Excel. The dots above bias indicate that the expression of genes was increased more than two folds and up-regulated 350 times, the dots under bias indicate that the expression of genes was decreased more than two folds and down-regulated 129 times, the dots between biases indicate that the expression of genes has no alteration. The expression of 59 genes was increased 2-128 folds, while the expression of 44 genes was increased 2-10 folds.



**Figure 2** Initial and total expression profiles of 85 innate immune response-associated genes at each time point of liver regeneration. Grey bars: up-regulated expression gene; white bars: down-regulated expression gene; blank bars: initially expressed genes in which up-regulated genes are predominant in the forepart, and down-regulated genes in the prophase and metaphase, whereas very few in the anaphase; dotted bars: the total number of expressed genes, in which the expression of some genes is up-regulated and the expression of others is down-regulated during LR.

whole LR. The expression of these genes was up-regulated 350 times and down-regulated 129 times. The expression of the genes was predominantly initially up-regulated in the forepart, and initially down-regulated in the prophase and metaphase, whereas the initial expression of very few genes was observed in the anaphase.

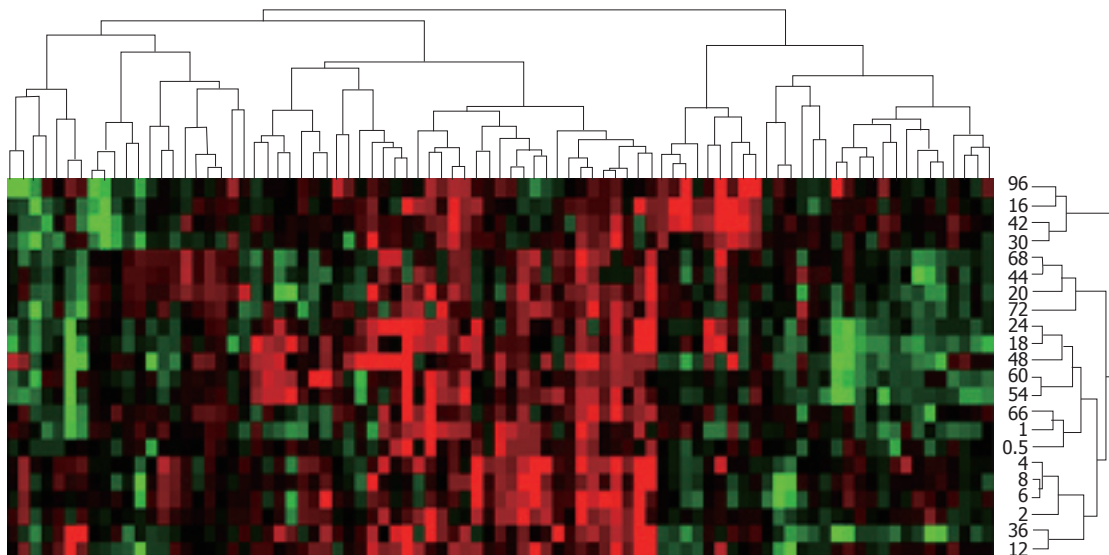
#### Expression similarity and time relevance of innate immune response-associated genes during liver regeneration

Based on their similar expression, the 85 genes during LR could be divided into 41 up-regulated, 4 predominantly up-regulated, 26 down-regulated, 6 predominantly down-regulated, and 8 up/down-regulated genes, respectively (Figure 3). Based on their time relevance, they could also be classified into 14 groups (0.5 h, 1 and 66 h, 2 h, 4 and 8 h, 12 and 36 h, 16 and 96 h, 18 and 24 h, 30 and 42 h, 48 h, 54 and 60 h, 72 h, 120 h, 144 and 168 h), in which their expression was up- and down-regulated at 10 and 2 h, 27 and 10 h, 18 and 1 h, 20 and 1 h, 30 and 8 h, 38 and 12 h, 33 and 11 h, 36 and 20 h, 26 and 12 h, 31 and 18 h, 10 and 4 h, 18 and 6 h, 26 and 11 h (Figure 3). The up-regulated expression of genes was mainly associated with cellular immunity. The down-regulated expression of genes was significantly associated with molecular immunity.

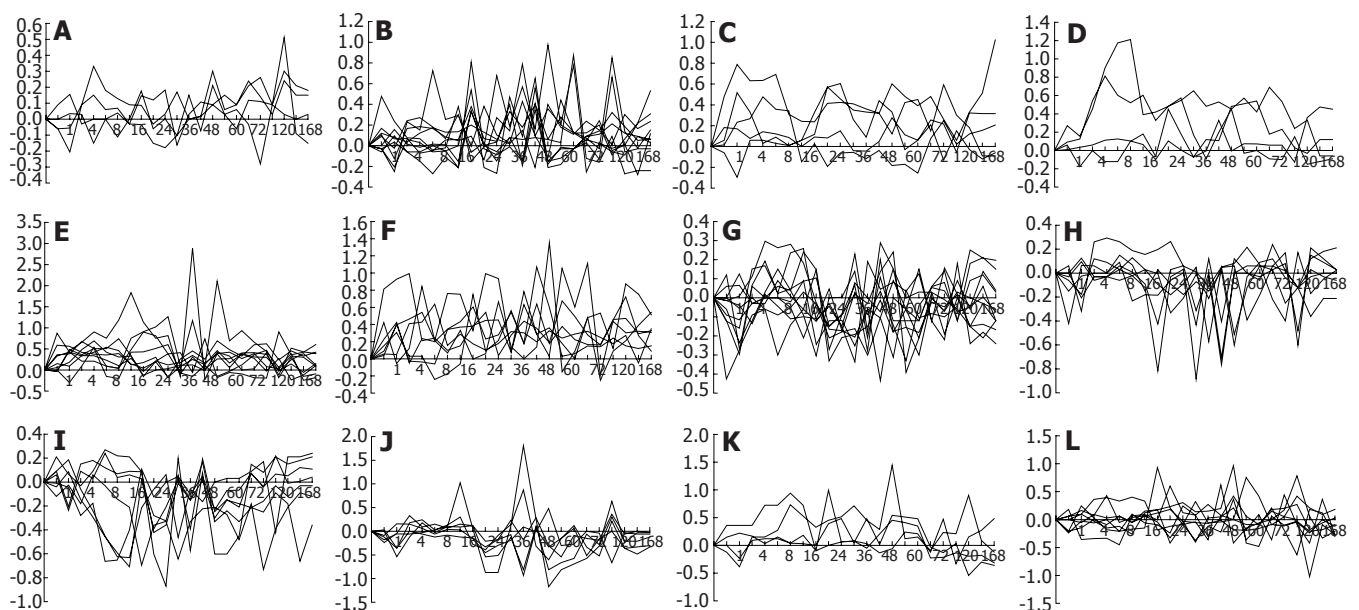
#### Expression patterns of innate immune response-associated genes during liver regeneration

According to their expression changes during LR, the patterns of the above 85 genes might be categorized into 28 types: 5 up-regulated genes at one time point, i.e. at 4, 48, 96, 120 h after PH (Figure 4A); 4 up-regulated genes at two time points, i.e. at 12 and 60 h, 42 and 120 h, 16 and 42 h (Figure 4B); 1 up-regulated gene at three time points (Figure 4B); 5 up-regulated genes at more time points (Figure 4B); 3 up-regulated genes at one phase, i.e. at 0.5-8 h, 4-8 h, 120-168 h (Figure 4C); 1 up-regulated gene at two phases, i.e. at 16-36 h, 42-48 h (Figure 4C); 1 up-regulated gene at three phases (Figure 4C); 1 up-regulated gene at more phases (Figure 4C); 1 up-regulated gene at one time point/one phase, i.e. at 120 and 2-72 h, 48 and 2-24 h, 18 and 48-60 h, 42 and 120-168 h (Figure 4D); 1 up-regulated gene at two time points/three phases (Figure 4E); 1 up-regulated gene at two time points/one phase (Figure 4E); 1 up-regulated at one time point/ three phases (Figure 4E); 1 up-regulated gene at two time points/three phases (Figure 4E); 3 up-regulated genes at two time points/two phases (Figure 4E); 1 up-regulated gene at three time points/one phase (Figure 4F); 1 up-regulated gene at three time points/two phases (Figure 4F); 3 up-regulated genes at





**Figure 3** Expression similarity and time relevance clusters of 85 innate immune response-associated genes during liver regeneration. Data detected by rat genome 230 2.0 array were analyzed by H-clustering. Red indicates up-regulated gene expression chiefly associated with cellular immunity; green indicates down-regulated gene expression mainly associated with molecular immunity; black indicates meaningless change in gene expression. The upper and right trees show the expression similarity and time series clusters, by which the above genes were classified into 5 and 14 groups respectively.



**Figure 4** Expression patterns of 85 innate immune response-associated genes during liver regeneration. Twenty-eight expression patterns were obtained by the analysis of data detected by rat genome 230 2.0 array with Microsoft Excel. **A-F**: 41 up-regulated genes; **G-I**: 26 down-regulated genes; **J-L**: 18 up/down-regulated genes. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

more phases (Figure 4F); 11 down-regulated genes at one time point, i.e. 0.5, 6, 16, 18, 30, 36, 42, 48, 54, 60 h (Figure 4G); 5 down-regulated genes at two time points, i.e. at 0.5 and 48, 1 and 72, 18 and 54, 30 and 42h (Figure 4H); 1 down-regulated gene at three time points (Figure 4H); 2 down-regulated genes at more time points (Figure 4H); 2 down-regulated genes at two time points/one phase (Figure 4I); 1 down-regulated gene at one phase, i.e. 6-12h (Figure 4I); 2 down-regulated genes at one time point/two phases (Figure 4I); 2 down-regulated genes at two time points/one phase (Figure 4I); 6 first down- and then up-regulated genes (Figure 4J); 4 first up- and then down-regulated genes (Figure 4K); 8 up/down-regulated genes (Figure 4L).

## DISCUSSION

Innate immune response, which is a self-protection mechanism formed during the long-term evolutionary process, includes tissue barrier, innate cellular immunity and innate molecular immunity, being closely linked to existence of high animal<sup>[2]</sup>. Of the proteins associated with innate cellular immunity, seven proteins including toll-like receptor 2 (TLR2) have a role in recognition of pathogens, interferon excretion of NK cells and activation of congenital immune system<sup>[24,25]</sup>; four proteins including attractin (ATRN) positively regulate antigen representation<sup>[26,27]</sup>; fifteen proteins including CCAAT/

enhancer binding protein beta (CEBPB) and S100 calcium binding protein A8 (S100A8) promote macrophage phagocytosis<sup>[28-32]</sup>; macrophage migration inhibitory factor (MIF), also called glycosylation-inhibiting factor, inhibits the function of macrophages<sup>[33]</sup>; glucocorticoid receptor (NR3C1) inhibits action of dendritic cells<sup>[34]</sup>. In the present study, the expression of the above genes was identical or similar at some time points, while different at other time points, indicating that they co-regulate cellular immune response. Among them, *cebpb* expression was up-regulated at 0.5-8 h after PH and reached its peak at 1 h, which was 3.1 folds of the control. *s100a8* expression was up-regulated at 2-72 h and 120 h after PH, and reached its peak at 4 h, which was 6.5 folds of the control. *nr3c1* and *thr2* expression was up-regulated at multiple phases during LR and reached their peak at 168 h and 42 h respectively, which was 4.7 folds and 10.6 folds of the control. *atrn* expression was up-regulated mainly at the metaphase, showing that the highest expression at 42 h was 4.4 folds that of the control. These findings suggest that the genes are the key to innate cellular immunity in regenerating liver.

Of the proteins associated with innate molecular immunity, eleven proteins, such as chemokine C-C motif ligand 2, 4 (CCL2, CCL4), are associated with recognition of pathogens and immune enhancement<sup>[35-38]</sup>. Two proteins, such as annan-binding lectin serine peptidase 1 (MASP1), activate the complement system<sup>[39]</sup>. Five proteins including complement component 2 (C2) enhance inflammation<sup>[40,41]</sup>. Four proteins including interferon regulatory factor 3 (IRF3) interfere with the multiplication of virus<sup>[42]</sup>. Two proteins including complement component 1 q subcomponent receptor 1 (C1QR1) are responsible for removal of apoptotic cells<sup>[43,44]</sup>. Ten proteins including interleukin 1 family member 5 delta (IL1F5) have a role in elimination of the pathogens in inflammatory response<sup>[45-47]</sup>. Three proteins including tumor necrosis factor (TNF) have the function of sterilization by promoting NK cell proliferation<sup>[48]</sup>. In the present study, the expression changes of the genes encoding these proteins were identical or similar at some time points and different at other time points, suggesting that they can co-modulate innate molecular immunity. *cc14* expression was up-regulated only at 48 h after PH, which was 3-fold of the control. However, Masson *et al.*<sup>[38]</sup> reported that it is down-regulated at 3 and 12 h. *cc12* expression was up-regulated at 0.5-1, 12-24, 36, 48-72 and 120 h, and reached its peak at 48 h, which was 128-folds of the control. *c1qr1* expression was up-regulated at multiple time points post PH and reached its peak at 8 h, which was 5.6 folds of the control. *masp1* expression was up-regulated at 42 and 120-168 h and reached its peak at 144 h, which was 3 folds that of the control. These findings indicate that the genes are important in molecular immunity during LR.

In conclusion, the expression changes of congenital immune response-associated genes after rat PH can be analyzed with high-throughput gene expression assay. The congenital immunity is enhanced during LR. Rat Genome 230 2.0 array was a useful tool analyzing the above response at transcriptional level. Nevertheless, DNA→mRNA→protein is influenced by various factors including protein interaction. Therefore, such techniques

as Northern blotting, protein chip, RNA interference, protein-interaction *etc.*, are needed to further test the above results.

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