Expression patterns and action analysis of the genes related to humoral immune response during rat liver regeneration☆

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Abstract

Objective. The aim of this project was to study humoral immune response after partial hepatectomy (PH) at transcriptional level. *Methods*. The genes involved in humoral immunity were obtained through the database data and the reference to the related thesis, and the gene expression changes in rat regenerating liver after PH were checked by Rat Genome 230 2.0 Array. *Results*. 67 genes were found to be associated with liver regeneration (LR). The initial and total expressing gene number occurring in initial phase of LR (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 23, 8, 38, 6 and 23, 18, 59, 44 respectively, illustrating that the associated genes were triggered mainly in initiation phase, and worked at different stages. The expression similarity of these genes were classified into only up-regulated, predominantly up-, only down-, predominantly down- and equally up- and down-, involving 26, 5, 23, 6 and 7 genes, respectively, and the total frequencies of up-regulation and down-regulation of these genes were 150 and 169, demonstrating that the up-regulated genes were slightly fewer than the down-regulated ones in number. Their time relevance was classified into 14 groups, showing that the cellular physiological and biochemical activities were staggered during LR. The expression patterns of them were classified into 20 types, demonstrating that the above activities were diverse and complicated during rat LR. *Conclusion*. The processes of antigen presentation and recognition were enhanced mainly in the forepart, late-metaphase and anaphase during LR; the level of immunocyte activation was increased mainly in the forepart and prophase; the enhancement of complement activation occurred in the forepart and anaphase. [Life Science Journal. 2007; 4(3): 49 – 56] (ISSN: 1097 – 8135).

Keywords: partial hepatectomy; Rat Genome 230 2.0 Array; humoral immune response; genes; liver regeneration

1 Introduction

After antigenic stimulus, antigens are taken, processed and presented to B lymphocytes by antigen-presenting cells, and then differentiation of specific B lymphocytes into plasma cells is triggered to release large amounts of secretive antibodies. Upon binding to their specific antibodies, the antigens are cleared by complement system[1]. The process above is called the humoral immune response. Humoral immune response is a kind of self-protective mechanism of organism developed during long evolutionary period and is key to high animal survival[2]. Usually, the immunoreactions were classified into cellular immunity and humoral immunity depending on the different effectors[3]. Humoral immune response works *via* the antigens produced by B lymphocyte. After partial hepatectomy (PH)[4], the damaged cells and cell remnants can severely harm to organism[5]; wound areas are also susceptible to infection by antigen and fremde stoffe[6]. Whether humoral immune system functions or not in this process deserves more intensive investigation.

At the same time, PH also activates the remnant hepatocytes to rapidly proliferate to compensate the lost liver tissue, which is called liver regeneration (LR)[7,8]. Generally, based on the cellular physiological activities, the regenerating process is divided into initial phase (0.5 – 4 h after PH), G0/G1 transition (4 – 6 h after PH), cell

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proliferation (6 – 66 h after PH), cell differentiation and structure-function reorganization (66 – 168 h after PH)[8]. According to time course, it is divided into 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)[9]. The above process involves many physiological and biochemical events such as cell activation, de-differentiation, proliferation and its regulation and re-differentiation[10] and is regulated by many factors including humoral immune response[11]. To study the relativity between the genes associated with humoral immune response and rat LR at the transcriptional level [9,12], the gene expression changes in regenerating liver after PH were detected by Rat Genome 230 2.0 Array[12] containing 121 humoral immune-associated genes[13], and 67 genes among them were identified to be related to LR[14]. And expression changes, patterns and actions of them were primarily analyzed.

2 Materials and Methods

2.1 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 at random and each group included 6 rats (male: female = 1:1). PH was performed according to Higgins and Anderson[4], 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, 24, 36, 54, 66, 72, 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, six samples of each group were gathered and mixed together to 1 – 2 g (0.1 – 0.2 g × 6) total liver tissue, then stored at – 80 °C. The sham-operation (SO) groups were the same with PH ones except that the liver lobes were not removed. The laws of animal protection of China were enforced strictly.

2.2 RNA isolation and purification

Total RNA was isolated from the frozen livers according to the manual of Trizol kit (Invittrogen)[15] and then purified base on the guide of RNeasy mini kit (Qiagen)[16]. In brief, the liver tissues frozen at – 80 °C were homogenized, splitting in Trizol reagent, chloroform extraction and isopentyl alcohol precipitation. The total RNA integrity was confirmed by agarose electrophoresis by checking the ratio of 28S RNA to 18S RNA. Total RNA concentration and purification were estimated by optical density measurements at 260/280 nm[17].

2.3 cDNA, cRNA synthesis and purification

As template, 1 – 8 μg total RNA was used for cDNA synthesis. cDNA purification was proceeded based on the way Affymetrix established[18]. cRNA labeled with biotin was synthesized using cDNA as the template and then purified[19]. Measurement of cDNA, cRNA concentration and purification were the same as above.

2.4 cRNA fragmentation and microarray detection

15 μl (1 μg/μl) cRNA incubated with 5 × fragmentation buffer at 94 °C for 35 min was digested into 35 – 200 bp fragments. Rat Genome 230 2.0 Array produced by Affymetrix was prehybridized, then the hybridization buffer added at 45 °C, 60 rpm for 16 h. 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[13].

2.5 Microarray data analysis

Signal values were quantified and normalized by GCOS 1.2 software. Quantified signal intensities were obtained by deducting foreground signal values. Signal intensities were replaced by the value 200 when they were < 200. When experiment/control (Ri) was between 0.1 and 10, Ri was taken as natural logarithms to generate lnRi and the normalize coefficient factor (ND) was taken by averaged Ri. The modified signal values were generated by ND multiplying control, and were replaced by the value 200 when it was < 200[13].

2.6 Normalization of the microarray data

To minimize error from the microarray analysis, each analysis was performed three times. Results with a total ratio was maximal (R^m) and that whose average of three housekeeping genes β-actin, hexokinase and glyseraldehyde-3-phosphate dehydrogenase approached 1.0 (R^h) were taken as a reference. The modified data was generated with 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 partial hepatectomy were reorganized by normalisation analysis program (NAP) software according to the cell cycle progression of the regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel software[20].

2.7 LR-associated genes identification

Firstly, the nomenclature of humoral immunity was adopted from the GENEONTOLOGY database (www.

geneontology. org), and inputted into humoral immune at NCBI (www. ncbi. nlm. nih. gov) and RGD (rgd. mcw. edu) to identify the rat, mouse and human genes associated with the activity mentioned above. According to maps of biological pathways embodied by GENMAPP (www.genmapp. org), KEGG (www. genome. jp/kegg/pathway. html#amino) and BIOCARTA (www. biocarta. com/genes/index. asp), the genes associated with humoral immune were collated. The results of this analysis were codified, and compared with the results obtained for human and mouse searches in order to identify human and mouse genes that 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 more than twofold change in expression level, observed as meaningful expression changes[13], were referred to as rat homologous genes or rat specific genes associated with humoral immune response under evaluation. Genes, which displayed reproducible results with three independent analysis with the chip and which showed morer than twofold change in expression level in at least one time point during LR with significant difference ($0.01 \leq P < 0.05$) or extremely significant difference ($P \leq 0.01$) between PH and SO, were referred to as associated with LR.

3 Results

3.1 General statement of the genes associated with humoral immune response during rat LR

According to the data of databases at NCBI, GENMAPP, KEGG, BIOCARTA and RGD, 357 genes were involved in humoral immune. In which, 121 genes were contained in the Rat Genome 230 2.0 Array. Among them, a total of 67 genes displayed meaningful expression changes at least at one time point after PH, showed significant or extremely significant differences in expression when comparing PH with SO, and displayed reproducible results with three detections with Rat Genome 230 2.0 Array, suggesting that the genes were associated with LR. The analysis indicated that 26 genes were up regulated, 23 genes down, and 18 genes up/down during LR. The range of up-regulation was from 2 to 128 times higher than control, and that of down-regulation was 2 – 20 folds (Table 1).

3.2 Expression changes of the expression of the humoral immune response-associated genes during rat LR

At each time point of LR, the numbers of initial up-, down regulated and total up-, down regulated genes were in sequence: both 3 and 5 at 0.5 h; 2, 5 and 3, 7 at 1 h; 2, 2 and 3, 3 at 2 h; 4, 0 and 10, 2 at 4 h; 2, 2 and 4, 6 at 6 h; 0 ,1 and 2, 6 at 8 h; 0, 2 and 4, 6 at 12 h; 8, 3 and 11, 6 at 16 h; 3, 7 and 9, 16 at 18 h; 0, 2 and 6, 11 at 24 h; 2, 1 and 11, 10 at 30 h; 0, 2and 5, 8 at 36 h; 1, 0 and 11, 6 at 42 h; 2, 0and 13, 11 at 48 h; 0, 0 and 4, 11 at 54 h; 0, 0 and 9, 8 at 60 h; 0, 0 and 6, 6 at 66 h; 0, 0 and 4, 6 at 72 h; 1, 0 and 13, 7 at 96 h; 3, 2 and 9, 14 at 120 h; 0, 0 and 6, 6 at 144 h; 0, 0 and 4, 8 at 168 h. In the respect of the initial expression of the above 67 genes, 33 and 34 genes were initially up regulated and down regulated during LR, respectively. A detailed introduction is as follows: at the initiation stage (0.5 – 4 h after PH), the G0/G1 transition phase (4 – 6 h after PH), cell proliferation period (6 – 66 h after PH), cell differentiation and the structure-function reorganization stage of LR (72 – 168 h after PH), the number of initially up and down regulated genes were 11 and 12, 6 and 2, 18 and 20, and 4 and 2. The overall situation of the genes expression was that 26 genes were up, 23 genes down, and 18 genes up/down regulated in regenerating liver and that total frequencies of up and down regulated expression were respectively 150 and 169. Specifically, at the above-mentioned four phases of LR, the number of times of up-regulation and down-regulation was separately 19 and 17, 14 and 8, 95 and 111, 42 and 47 (Figure 1).

3.3 Expression similarity and time relevance of the humoral immune response-associated genes during rat LR

67 genes mentioned above could be characterized based on their similarity in expression as following: only up, predominantly up, only down, predominantly down and up/down regulated, involved in 26, 5, 23, 6 and 7 genes, respectively (Figure 2). According to time relevance, they were classified into 14 groups, including 0.5 and 48 h, 1 h, 2 and 4 h, 6 h, 8 and 12 h, 16 h, 18 and 24 h, 30 and 42 h, 36 and 54 h, 60 h, 66 and 72 h, 96 h, 120 h, 144 and 168 h, and the up and down regulated times were 3 and 5, 7 and 18, 3 and 3, 14 and 8, 2 and 6, 15 and 12, 9 and 16, 17 and 21, 18 and 19, 20 and 14, 6 and 6, 17 and 13, 9 and 14, 10 and 14, respectively (Figure 2). The up-regulation expression genes were chiefly associated with antigen processing and presentation and immunocyte activation and the down-regulation ones were mostly complement activation-associated.

3.4 Expression patterns of the humoral immune response-associated genes during rat LR

67 genes mentioned above were categorized into 20 patterns, according to the changes in their expression.(1) up-regulation at one time point, i.e. 4, 18, 30, 48, 96, 120 h after partial hepatectomy (Figure 3A), 8 genes involved;

Table 1. Expression abundance of 67 humoral immune response-associated genes during rat liver regeneration

Name	Abbr.	Associated to others	Fold difference	Name	Abbr.	Associated to others	Fold difference
1 Antigen processing and presentation				chemokine ligand 2	Cd2		128.0
dermatopontin	Dpt		0.4	interleukin 4	Ii4		0.1, 2.6
activated leukocyte cell adhesion molecule	Alcam		0.5	POU domain class 2 associating factor 1	Pou2af1		0.1, 3.4
petidylprolyl isomerase A	Ppia		2.6	chemokine C motif ligand 1	Xcl1		0.1, 3.7
tumor necrosisfactor receptor superfamily member 17	Tnfrsf17		3.2	paired-Ig-like receptor B	Pirb		0.2, 7.0
complement receptor 2	Cr2	3	6	complement component 3a receptor 1	C3ar1	3	0.3, 2.3
immunoglobulin heavy chain epsilon polypeptide	Ighe		0.2, 2.5	cluster of differentiation 2 antigen	Cd2		0.3, 2.6
chemokine c-c motif receptor 6	Ccr6		0.3, 43	interleukin 2	Ii2		0.3, 3.5
ubiquitin D	Ubd		0.3, 5.7	nuclear factor of kappa light hene enhancer in B-cells 1 p105	Nikb1		0.4, 2.3
basic leucine zipper transscription factor ATF-like	Batf		0.4, 4.9	complement component 5 receptor 1	C5r1	3	0.4, 2.6
2 Immunocyte activation				membrance-spanning 4-domains subfamily Amember 2	Ms4a2		0.4, 5.3
complement component 2	C3	1	0.2	nuclear receptor sunfamily 4 group A member 2	Nr4a2		0.4, 7.1
cluster of differentiation 83 antigen	Cd83		0.2	**3 Comolement activation**			
cluster of differentiation 24 antigen	Cd24		0.2	adenosine deaminase	Ada		0.1
cysteine-rich protein 1	Crip 1		0.2	proteolipid protein	Plp		0.1
Epstein-Barr virus induced gene 3	Ebi3		0.2	complement component 3	C3	2	0.2
immunoglobulin joining chain	Igj		0.3	decay accelarating factor 1	Daf1		0.2
inositol polyphosphate-5-phosphatase D	Inpp5d		0.3	mannose binding lectin 2	Mbl2		0.2
mitogen activated protein kinase 11	Mapk11		0.3	complement component 1 q subcomponent alpha	C1qa		0.3
bone marrow stromal cell antigen 2	Bst2		0.4	immunoglobulin heavy chain 1a	Igh1a		0.3
cluster of differentiation 22 antigen	Cd22		0.4	complement component 4a	C4a		0.5
cluster of differentiation 74 antigen	Cd74		0.4	cluster of differentiation 59 antigen	Cd59		0.5
integrin beta 2	Itgb2		0.5	complement component 4 binding protein alpha	C4bpa		2.0
microsomal glutathione S-transferase 3	Mgst3		0.5	complement component 2	C2		2.1
polycomb group ring finger 4	Pcgf4		2.0	B-cell leukemia/lymphoma 10	Bcl10		2.3
myeloid differentiation primary response gene 88	Myd88		2.1	complement receptor related protein	Crry		2.4
cytochrome b-245 beta	Cybb		2.5	complement factor H	Cfh		2.5
cluster of differentiation 86 antigen	Cd86		2.6	clusterin	Clu		3.0
interleukin 7	Ii7		2.8	mannan-binding lectin serine peptidase 1	Masp1		3.0
tumor necrosis factor	Tnf		3.2	complement receptor 2	Cr2	1	6.0
cluster of differentiation 79B antigen	Cd79b		3.5	complement factor I	Cfi		6.4
prostaglandin E synthase	Ptges		4.5	mannose binding lectin 1 protein A	Mbl1		0.2, 2.1
mitogen activated protein kinase 13	Mapk13		6.1	complement component 3a receptor 1	C3ar1	2	0.3, 2.3
platelet-activating factor receptor	Ptafr		7.1	enolase 1	*Eno1		0.3, 2.3
chemokine ligand 20	Ccl2o		8.0	myelin-associated glycoprotein	*Mag		0.4, 2.3
colony stimulating factor 1 receptor	Csf1r		13.0	complement component 5 receptor 1	C5r1	2	0.4, 2.6
chemokine C-C motif ligand 7	Ccl7		22.6				

*Reported genes associated with lLR; Associated to others: involved in other responses.

(2) up at two time points, i.e. 16 and 42 h, 48 and 120 h (Figure 3B), 2 genes involved; (3) up at four time points (Figure 3B), 4 genes involved; (4) up at one phase, i.e. 4 – 8, 120 – 168 h (Figure 3B), 3 genes involved; (5) up at one time point/phase, i.e. 0.5 and 4 – 12 h, 1 and 66 – 72 h, 42 and 120 – 168 h (Figure 3B), 3 genes involved; (6) up at two time points/ phases (Figure 3B), 2 genes involved; (7) up at more time points/more phases (Figure 3C), 5 genes involved; (8) down at one time point, i.e. 8, 12, 16, 18, 36, 120 h (Figure 3D), 6 genes involved; (9) down at two time points, i.e. 0.5 and 48 h, 36 and 48 h, 120 and 168 h (Figure 3D), 3 genes involved; (10) down at one time point/phase, i.e. 42 and 24 – 30 h, 54 and 18 –24 h, 60 and 18 – 24 h (Figure 3D), 3 genes involved; (11) down at one time point/three phases (Figure 3E), 2 gene involved; (12) down at two time points/one phase (Figure 3E), 2 genes involved; (13) down at two time points/ phases (Figure 3E), 2 gene involved; (14) down at more time points/more phases (Figure 3F), 5 gene involved; (15) predominantly up (Figure 3G), 5 genes involved; (16) predominantly down (Figure 3G), 6 genes involved; (20) similarly up/down (Figure 3H), 7 genes involved.

4 Discussion

Humoral immunity refers to antibody production, and the processes include antigen processing and presentation,

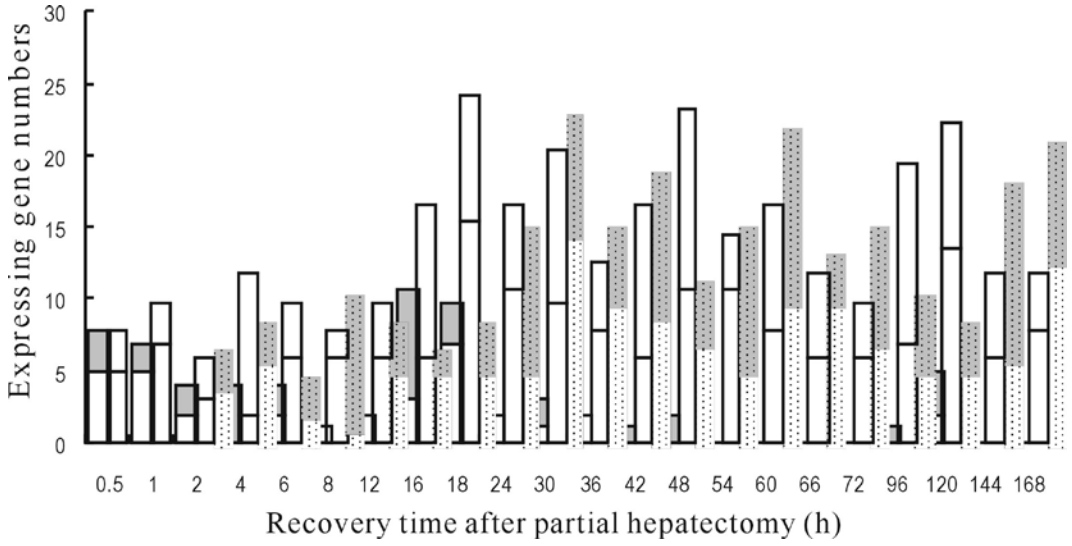

Figure 1. The initial and total expression profiles of 67 humoral immune response-associated genes at each time point of liver regeneration. Blank bars: Initially expressing gene number; Dotted bars: Total expressing gene number; Grey-background bars: Up regulated genes; White-background bars: Down regulated genes. Expression change of the genes spans the whole live regeneration. Initially up regulated genes are predominated at 4 – 6, 16, 30, 42 – 48 and 96 – 120 h after PH; there are no initially expressed genes at 54 – 72 and 144 – 168 h.

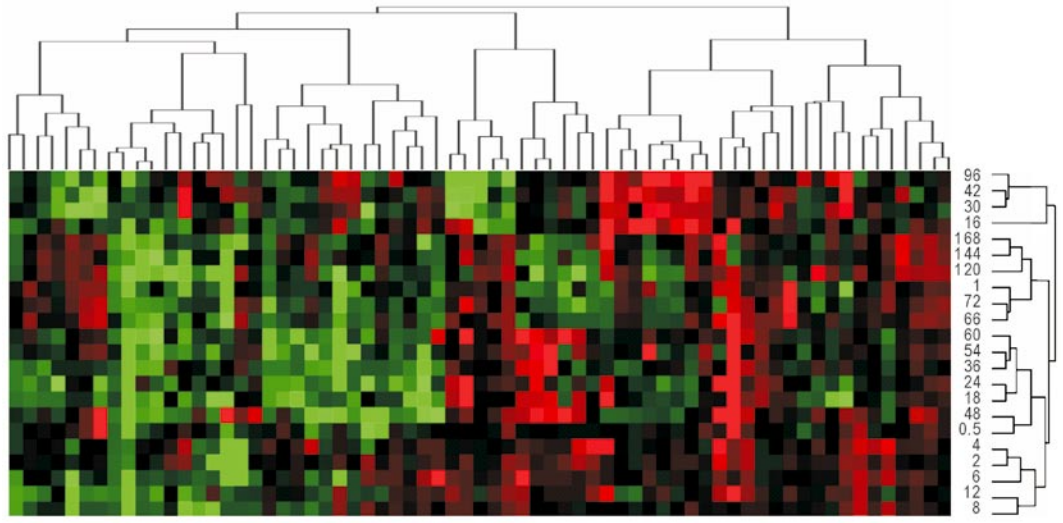

Figure 2. Expression similarity and time relevance clusters of 67 humoral immune response-associated genes during LR. Detection data of Rat Genome 230 2.0 Array were analyzed by H-clustering. Red represents up-regulation genes chiefly associated with antigen processing and presentation, immunocyte activation; Green represents down-regulation ones mainly associated with complement activation; Black: No-sense in expression change. The upper and right trees respectively show expression similarity cluster and time relevance cluster, by which the above genes were classified into 5 and 14 groups separately.

immunocyte activation and cytokine production, as well as classical complement activation. Humoral immunity is called as such because of the involvement of amounts of substances (proteins) found in body fluids. As a typical course, rat liver regenerating process also involves above physiological activity. Among the proteins participating in antigen processing and presentation, three proteins including CR2 promote antigen presentation[21], UBD accelerates antigen processing[22]; four proteins including BATF play a role in antigen recognition[23]; ALCAM can expedite immunocyte migration and aggregation[24]. The meaningful expression profiles of the genes encoding these cytokines are same or similar at some points while different at others, indicating that they may regulate an-

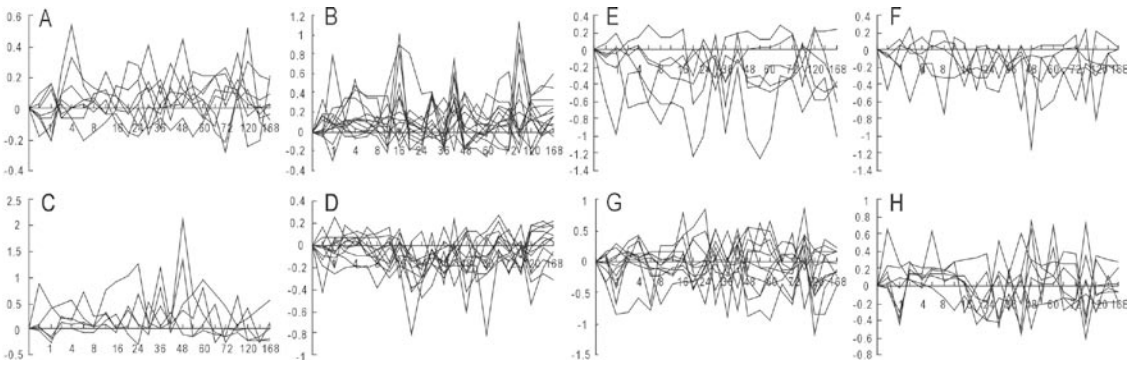

Figure 3. Twenty gene expression patterns of 67 humoral immune response-associated genes during LR. Expression patterns were obtained by the analysis of detection data of Rat Genome 230 2.0 Array with Microsoft Excel. A – C: 26 up regulated genes; D – F: 23 down regulated genes; G – H: 18 up/down regulated 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.

tigen presentation, processing and recognition together in LR. Among them, the up regulated gene *ubd* mainly at 0.5, 48, 66 – 72 and 120 h after PH reached its climax with 5.7-fold of control at 48 h; the increased expression of *batf* mainly at 48 and 60 h exhibited peak value of 4.9 fold of control at 60 h; *cr*2 was up at 6, 18, 36, 48 and 60 h, and reached a peak at 60 h that was 6-fold of control. It was presumed that they maybe function in or reflect antigen processing and presentation during LR.

Of immunocyte activation-associated proteins, six proteins including PTAFR are capable of stimulating immunocyte activation[25]; fourteen proteins including NR4A2 can promote activation, differentiation, proliferation and maturity of leukomonocyte including T cell and B cell[26]. C3AR1 and C5R1 accelerate pyrolysis of the target cell by promoting leukomonocyte differentiation and the migration of lymphocyte toward inflammation sites[27,28]; TNF and CCL7 induce the movement of immunocytes towards inflammatory sites[29,30]; EBI3 accelerates the immune response mediated by Th2 whose generation may be positively regulated by IL4[31,32]; PIRB participates in B cell-mediating humoral immunity[33]; IGJ maintains the stability of the total mass of IgA[34]; the binding of MS4A2 to IgE β chain can lead to anaphylaxis[35]; XCL1 enhances the lymphocyte chemotactic response[36]; NFKB1 is able to positively regulate the expression of chemokine[37]; three proteins including CCL2 increase antigen activity and promote antigen-antibody binding [38]; both CYBB and ITGB2 can enhance phagocytosis activity of immunocyte[39,40]; the excessive expression of CD22 and CD24 can induce autoimmunity[41,42]. The meaningful expression profiles of the genes encoding above proteins were same or similar at some points while different at others, indicating that they may co-regulate immunocyte activation. Particularly deserving mention are the following genes: the up-regulated genes *ptafr* and *nr*4*a*2 at multiple time points after PH both reached their peak with 7.1 times higher than the control at 96 h; *pirb* was up-expressed at 16 – 24, 60, 72 and 144 h, and reached a peak at 24 h, that was 7 times higher than the control; *ccl*7 up at 1 – 4, 12, 24, 48, 66 and 144 – 168 h, and peaked the highest abundance with 22.6 folds of control at 48 h; *ccl*2 expression was increased at 0.5 – 1, 12 – 24, 36, 48 – 72 and 120 h, reaching a peak with 128 times higher than control at 48 h. The above results maybe imply that these genes are important in immunocyte activation during LR.

Among complement activation-related proteins, four proteins including PLP promote complement activation[43], while CLU and CRRY functioned oppositely[44,45]; four proteins including C2 are the important components of the classical pathway of complement activation[46]; IGH1A regulates antibody responses by activating the Fcγ or FcγII B receptor[47]; CFH activates complement bypass[48]; CFI can inactivate the complement by hydrolyzing C3b and C4b[49]. C4BPA and MASP1 are the important ingredients of complement system[50,51]; C3 maybe accelerates lysis[52]; BCL10 is the well-known apoptosis-promoting protein[53]. The meaningful expression profiles of the genes encoding these proteins are same or similar at some points while different at others, presuming that they may co-regulate complement activation. Notably, the up regulated gene *cfi* in LR reached a peak (6.4 times than the control) at 16 h, probably implying its critical role in complement activation during LR.

In conclusion, the expression changes of the humoral immunity-associated genes after PH were investigated using high-throughput gene expression analysis. It was primarily proved that humoral immune response is enhanced

in regenerating liver; that Rat Genome 230 2.0 Array is a useful tool analyzing the response at the transcriptional level. However, the processes of DNA→mRNA→protein→function were affected by many factors including proteins interaction. Therefore, we will further analysis the above results by the techniques, such as Northern blotting, protein chip, RNA interference, protein-interaction etc.

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