

Comparative Analysis of the Role of ERK1/2 Signaling Pathway in Regulating Cell Proliferation of Rat Liver Regeneration and Rat Acute Hepatic Failure

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Abstract

After injured or partially hepatectomy (PH), hepatocytes could rapidly enter cell cycle to compensate for the loss of liver tissue, which is regulated by lots of signaling pathway, including ERK1/2 signaling pathway. To compare the role of ERK1/2 signaling pathway in PH-induced liver regeneration (LR) with that in carbon tetrachloride-induced acute hepatic failure (AHF) at gene transcription level, Rat Genome 230 2.0 array was used to detect gene expression profiles of above two processes, and bioinformatics and systems biology methods were applied to analyze the physiological activities uncovered by their profiles. The results showed that 165 genes were associated with fourteen paths of ERK1/2 signaling pathway, 161 genes were contained in the array above, and 46 genes were significantly expressed. Of them, 36 genes were the LR-specific genes, 24 genes were the AHF-specific, and 14 genes were the common genes. Synergy value of these genes was calculated using a

mathematical model established by our lab, and the results showed that the cell proliferation-promoting effect of path 4 was weaker than the control at 6h of AHF occurrence, while those of paths 2, 8, and 9 at 12h of LR and paths 2 and 6 at 72h of AHF were stronger. On the other hand, the cell proliferation-inhibiting effect of path 11 was stronger at 12h of LR and AHF, but that of path 13 was weaker at 24h of LR. In conclusion, 46 genes related to seven paths of ERK1/2 signaling pathway regulates cell proliferation in both LR and AHF.

Keywords: Rat liver regeneration, Rat acute hepatic failure, ERK1/2 signaling pathway, Rat Genome 2302.0 Array, Cell proliferation

1. Introduction

The liver has a lot of important physiological functions and a strong ability to regenerate (Yokoyama et al., 2007). After injured or partially hepatectomy (PH), it can rapidly restore to the normal mass via proliferation and growth of the remnant liver, which is called liver regeneration (LR) (Higgins et al., 1931). This process is regulated by many signaling pathways including ERK1/2 signaling pathway. It was found that lots of physiological activities, including activation of cytokines and growth factors, synthesis and activation of transcription factors and enzymes, and synthesis and reconstruction of extracellular matrix were related to LR (Guo et al., 2006; Xu et al., 2010; Kant et al., 2006). In 2/3 hepatectomy-induced rat regenerating liver, hepatocytes were activated at 2h after PH, their transition from G0 phase to cell cycle occurred at 2-6h after PH, their G1 phase, S phase, G2 phase, and M phase of first cell cycle were 6-12h, 12-24h, 24-30h, and 30-36h after PH, respectively, and their second cell cycle was 36-66h after PH (Xu et al., 2010). Other types of hepatic cells also carried out proliferation and growth during this period. Then, the structure and function of the regenerating liver was re-established, and LR is completed within one week after PH.

Acute hepatic failure (AHF) is a kind of rapid liver cell necrosis or severe impairment of liver function which was induced by pathogenic factors. Lots of physiological activities including inflammatory and immune responses, signal transduction, metabolism, oxidation reduction, cell proliferation, differentiation and apoptosis occurred in AHF occurrence, which is regulated by many signaling pathways including ERK1/2 signaling pathway. Carbon tetrachloride (CCl₄)-induced AHF was divided into injury phase (3-6h after CCl₄ treatment), progressing phase (12-24h after CCl₄ treatment) and recovery phase (48-72h after CCl₄ treatment) (Mookerjee et al., 2007; Felipo et al., 2002; Cheng et al., 2010). At 3h after CCl₄ treatment, cytoplasm of hepatocytes became loose, and hydropic degeneration occurred to some. At 6h after CCl₄ treatment, hepatocytes underwent light vesicles-like steatosis, the hepatic sinusoids were hyperemic with obvious inflammatory cells infiltration. At 12h after CCl₄ treatment, hepatic lobes were damaged with obvious steatosis of hepatocytes, and hyperemia in hepatic sinusoids became more evident. At 24h after CCl₄ treatment, hepatic lobes injury increased with evident flake-shaped necrosis and monocytes and lymphocytes infiltration. In addition, hepatocytes underwent balloon sample degeneration, and hepatic sinusoids congestion became serious. At 72h after CCl₄ treatment, little lake-shaped but not bridge-shaped necrosis was observed in interlobular and portal area, hepatocyte proliferation and differentiation increased, and hepatic lobes recovered obviously.

ERK1/2 signaling pathway can regulate lots of physiological activities including cell proliferation and differentiation. After PH or drug injured, a large number of hepatocytes rapidly enter cell cycle to compensate for the loss of liver tissue, this process is regulated by lots of signaling pathway, including ERK1/2 signaling pathway. And ERK1/2 signaling pathway is composed of many proliferation-regulating paths. Our lab subdivided the ERK1/2 signaling pathway into 14 branch pathways which were named "paths", according to the path-path relevance and the role of the path, and according to the MEK1/2-activating factors, these paths were further grouped into four kinds. In the first kind of paths including path 1, 7-9 and 11, hormones and neurotransmitters activated G-protein, then MEK1/2. In the second kind of path, path 2, the concentration of Calcium ion varied, activating MEK1/2. In the third kind of paths, path 3-4, cytokines and growth factors combined with tyrosine kinase receptor, activating MEK1/2. In the fourth kind of paths, path 5-6, extracellular matrix stimulated integrin-protein, activating MEK1/2. These factors assembled signals into MAPK kinase cascade, that is, MAPKKK (Raf) → MAPKK (MEK1/2) → MAPK (ERK1/2), activating ERK1/2. The activity of ERK1/2 was also regulated by other kinases (Path 10, 12-14) (Figure 2). Activated ERK1/2 enters the nuclear and phosphorylate lots of transcription factors and nuclear proteins to promote cell proliferation etc.

Several groups have reported that PH could activate ERK1/2 (Fausto et al., 2000; Huh et al., 2004; Leu et al., 2003), and ERK1/2 signaling could promote cell proliferation through regulating cell cycle progression in LR (Murata et al., 2007; Molecular And Cellular Biology et al., 1999; Rescan et al., 2001), and further promote the recovery of liver injury. Our lab has also compared expression profiles and role of rat LR-related genes with those of rat AHF-related genes at liver tissue level. However, it is unclear whether these paths participate in the

regulation on liver regeneration, and how they do if any. To further explore the role of ERK1/2 signaling pathway in regulating rat LR and rat AHF at the gene transcription level, Rat Genome 2302.0 array was used to detect the expression changes of ERK1/2 signaling pathway-related genes in rat LR and in rat AHF, and bioinformatics and systems biology methods to analyze the influence of these changes on cell proliferation and apoptosis of rat LR and rat AHF occurrence.

2. Materials and Methods

2.1 Rat 2/3 hepatectomy and rat acute hepatic failure

Sprague-Dawley (SD) rats were obtained from the experimental Animal Center of Henan Normal University. They were bred in standard laboratory conditions (temperature 21 ± 2 °C, relative humidity $60\%\pm 10\%$, and 12:12 h light–dark cycle with lights on 8:00–20:00). 114 healthy SD rats, each weighting 230 ± 20 g, were picked up and randomly divided into 9 partial hepatectomy groups, 9 operation control (OC) groups and one normal control (NC) group. Each group contained 6 ones. Rats in PH groups underwent 2/3 PH as Higgins et al (1931) described, and they were sacrificed to isolate the liver tissues at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168 hours after PH. In addition, 28 male rats, each weighting 250–320g, were picked up and randomly divided into 7 groups with 4 rats in each including 6 rat acute hepatic failure (AHF) group with 24 rats, and one control group with 4 rats. The rats were fed with standard rodent chow diet with free access to distilled water. The rats in AHF were fed with a single dose of 4ml/kg carbon tetrachloride (CCl₄) which was diluted 2:3 in sesame oil, and 4 livers were taken for use at each of the following time points: 3, 6, 12, 24, 48 and 72 hours after CCl₄ treatment. Livers of control group were taken at 0h. The whole handling procedure went in accordance with the current Animal Protection Law of China.

2.2 Rat Genome 230 2.0 microarray detection and data analysis

Take 0.5g liver tissues from the middle part of right lobe. Their total RNA was extracted from the frozen regenerating livers with Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA) following its manufacturer's instructions and purified following the RNeasy mini protocol (Qiagen, Inc, Valencia, CA, USA), respectively. The quality of total RNA samples was assessed by measuring the optical density at 260/280nm and agarose electrophoresis (180 V, 0.5 h) with a 2:1 ratio of 28S rRNA to 18S rRNA intensity. As a template, 5 µg of total RNA was used to synthesize the first strand of cDNA using SuperScript II RT (Invitrogen Corporation, Carlsbad, CA), and T7-oligo dT(24) (W.M. Keck Foundation, New Haven, CT) as the primer. Second strand synthesis was performed with the Affymetrix cDNA single-stranded cDNA synthesis kit. The cDNA product was purified following the cDNA purify protocol. Twelve µL purified cDNA served as a template for the production of biotin-labeled cRNA transcript using the GeneChip in vitro Transcript Labeling Kit (ENZO Biochemical, New York, NY). The labeled cRNA was purified using the RNeasy mini columns (Qiagen, Valencia, CA) [7]. The concentration, purity and quality of cDNA and cRNA were assessed as above. Fifteen µL cRNA (1µg/µL) was incubated with 6µL 5×fragmentation buffer and 9µL RNase free water for 35 min at 94 °C, and digested into 35–200 bp cRNA fragments. The prehybridized rat genome 230 2.0 microarray was put into a hybridization buffer prepared following the Affymetrix protocol, and hybridized in a rotating chamber (60 rpm, 16 h, 45 °C). Arrays were washed to remove the superfluous hybridization buffer, stained in GeneChip fluidics station 450 (Affymetrix Inc., Santa Clara, CA, USA), scanned with a GeneChip scanner 3000 (Affymetrix Inc., Santa Clara, CA, USA) to obtain images (Xu et al., 2008).

2.3 Quantitative real-time RT-PCR

To verify the chip data, seven genes were selected for real-time RT-PCR analysis. The primer sequences were designed by Primer Express 2.0 software according to seven target genes *a2m*, *spp1*, *cyp11a1*, *jun*, *icam1*, *g6pc*, and *trim24* (GenBank number: NM_012488, NM_012881, NM_012540, NM_021835, NM_012967, NM_013098 and NM_001044266) and synthesized by Shanghai Generay Biotech Co., Ltd. The gene-specific primers were following: forward primer 5'-CGAACATCCGTAACCCAAAGTC-3' and reverse primer 5'-GCTGAGTCCACCACCACCAA-GTC-3' for *a2m*, forward primer 5'-TGATGACGACGACGATGACGATGG-3' and reverse primer 5'-ACGCTGGGCAACTGGGATGACCTT-3' for *spp1*, forward primer 5'-AGGACAGGAGGCT-GGACGAGA-3' and reverse primer 5'-ATGGTGAATGGGACAAAGGAT-3' for *cyp11a1*, forward primer 5'-TGCAAAGATGGAAACGACCTT-3' and reverse primer 5'-GCCGTAGGCGCCACTCT-3' for *jun*, forward primer 5'-TCAAACGGGAGATGAATGGT-3' and reverse primer 5'-CCTCTGGCG-GTAATAGGTGT-3' for *icam1*, and forward primer 5'-CTCAGGAACGCCTTCTATG-3' and reverse primer 5'-ACGGAGCTGTTGCTGTAAT-3' for *g6pc*, forward primer 5'-CAGTGGGAGGGTCTTA-CAATC-3' and reverse primer 5'-CTGGCCAGGGTCTACTTGT-3' for *trim24*. Firstly their total mRNA of liver tissues from

regenerating liver was isolated at the 10 recovery time points. Of them, 2 μ g RNA was reverse-transcribed using random primers and reverse transcription kit (Promega). First-strand cDNA samples were subjected to quantitative PCR amplification by using SYBR® Green I on the Rotor-Gene 3000A (Corbett Robotics, Brisbane, Australia). All of the PCR cycling conditions were modified at 95°C for 2 min, followed by 40 cycles of 95°C for 15s, 60°C for 15s, and 72°C for 30s. Every sample was analyzed in triplicate. Standard curves were generated from five repeated ten-fold serial dilutions of cDNA, and the copy numbers of target genes in every milliliter of the sample were calculated according to their corresponding standard curves (Wang et al., 2010).

2.4 Identification of rat liver regeneration-related genes and rat acute hepatic failure-related genes in ERK1/2 signaling pathway

To identify genes associated with ERK1/2 signaling pathway, the phrase “ERK1/2 signaling pathway” was input into NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to collect its genes associated with rat, mouse and human. Then, these genes were further obtained according to the biological pathway maps embodied at the databases including GENMAPP (www.genmapp.org), KEGG (www.genome.jp/kegg/pathway.html) and BIOCARTA (www.biocarta.com/genes/index.asp), etc and reconfirmed with related articles (Wang et al., 2007; Doniger et al., 2003; Ogata et al., 1999; Osband et al., 1990). In confirming the genes related to rat liver regeneration (LR) and the genes related to AHF, the images above were converted into signal values using Affymetrix GCOS 1.4 software. The data of each array were initially normalized by scaling all signals to a target intensity of 200. When P value is < 0.05 , it means that the gene is present (P), when < 0.065 , means marginal (M), and when > 0.065 , means marked absent (A). Then, the signal value of each chip was normalized, and the relative values of genes were evaluated with the ratios comparing the normalized P value in PH groups to that in control groups. For example, the expression of genes with a relative value ≥ 3 is considered up-regulated, the expression of genes with a relative value ≤ 0.33 is considered as down-regulated, and genes with a relative value of 0.33~2.99 are considered as unmeaning genes. To minimize the technical errors from microarray analysis, each sample was analyzed at least three times with Rat Genome 230 2.0 microarray. Their average value was calculated as a corrective value. The genes which have expressed significantly during CCl₄-induced AHF were considered as AHF-related genes, and the genes which have been significantly changed during LR with significant difference ($0.01 \leq P < 0.05$) or extremely significant difference ($P \leq 0.01$) between PH and OC, as LR-related genes.

2.5 Synergy analysis of ERK1/2 signaling pathway-related genes

A mathematical model (E_t) was established to describe how physiological activities are governed by gene synergy (Xu & Zhang, 2009), in accordance with the gene expression abundance of ERK1/2 signaling pathway in regenerating liver tissues detected by Rat Genome 230 2.0 Array, the multivariate statistics (Chalmers et al., 1998), time series analysis (McCubrey et al., 2007), correlation analysis (r_{ik}) (Liu et al., 2009), and the principle that gene synergy governs physiological activity:

$$E_t = \frac{\sum_{i=1}^n \sum_{k=1}^n [(X_i^{(t)} + X_k^{(t)}) * r_{ik}]}{n(n+1)}$$

In the formula, the genes correlation coefficient (r_{ik}) was defined as:

$$r_{ik} = \frac{m(\sum_{t=1}^m X_i^{(t)} X_k^{(t)}) - (\sum_{t=1}^m X_i^{(t)}) (\sum_{t=1}^m X_k^{(t)})}{\sqrt{[m \sum_{t=1}^m X_i^{(t)2} - (\sum_{t=1}^m X_i^{(t)})^2] [m \sum_{t=1}^m X_k^{(t)2} - (\sum_{t=1}^m X_k^{(t)})^2]}}$$

“E” means gene synergy value which refers to physiological activity. “t” represents orders time series. “ $X_i^{(t)}$ ” and “ $X_k^{(t)}$ ” abundances of gene at t. “n” the total number of genes. Physiological activities in LR are more strengthened than in control when $E_t - E_0 > E_0$, more weakened when $E_t - E_0 < 0$, similar in the two groups when $E_t - E_0 = 0$.

3. Results

3.1 The expression profiles of ERK1/2 signaling pathway-related genes in rat liver regeneration and in rat acute hepatic failure

The data from NCBI, RGD, etc and biological pathway maps in GENMAPP, KEGG and BIOCARTA etc showed that 165 genes were related to ERK1/2 signaling pathway. Of them, 161 genes were in Rat Genome 230 2.0 Array. This array was used to detect expression changes of genes related to ERK1/2 signaling pathway in rat liver regeneration (LR) and in rat acute hepatic failure (AHF), showing that 46 genes were significantly expressed. In detail, 36 genes were the LR-specific genes, including 29 up-regulated and 7 down-regulated genes. 24 genes were the AHF-specific genes, including 18 up-regulated and 6 down-regulated genes. And 14 genes were the common genes. Of them, 9 genes were up-regulated and 2 genes were down-regulated in both LR and AHF, 2 genes were up-regulated in LR and down-regulated in AHF, one gene was down-regulated in LR and up-regulated in AHF (Table 1, Figure 1). To confirm the reliability of the array detection results, real-time RT-PCR was used to detect the expression changes of genes including *a2m*, *spp1*, *cyp11a1* and *trim24* in LR and AHF, and it was founded that the gene expression trends of these genes detected by RT-PCR and Rat Genome 230 2.0 Array were generally consistent, suggesting that the array check results were reliable.

3.2 Comparative analysis of the detection results of real-time polymerase chain reaction and that of microarray

To confirm the results of the microarray analysis, RT-PCR assays were used to detect the expression changes of the genes including *a2m*, *spp1*, *cyp11a1* and *trim24* at 3, 6, 12, 24, 48 and 72h after CCl₄ treatment and *jun*, *icam1*, *g6pc*, and *trim24* in regenerating livers at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168h after PH. The result showed that relative abundance of mRNA were not all the same, but expression trends of the genes detected by above two methods were generally consistent, suggesting that the array detection results were reliable (Figure 2).

3.3 The physiological activities uncovered by expression profiles of ERK1/2 signaling pathway-related genes in rat liver regeneration and rat acute hepatic failure

The gene synergy and the roles of the fourteen paths of ERK1/2 signaling pathway in rat LR and rat AHF were analyzed by using the mathematical model above, and the results were listed blow. Gene synergy value (E_t) of cell proliferation-promoting path 4 was smaller than that of control at 6h of rat AHF occurrence, and that of path 13 was smaller at 24h of rat LR. But E_t of cell proliferation-promoting paths 2, 8, 9 and 11 were greater at 12h of rat LR, and E_t of cell proliferation-promoting path 11 were greater at 12h of rat AHF occurrence. E_t of all paths were similar to that of the control at 24h of rat AHF occurrence and 6h, 72h of rat LR. E_t of cell proliferation-promoting paths 2 and 6 were greater at 12h of rat AHF occurrence. However, paths 1, 3-5, 7, 10, 12 and 14 were not found to participate in LR and AHF occurrence (Figure 3).

4. Discussion

It was reported that 2-6h after rat partial hepatectomy (PH) is the priming phase of rat liver regeneration (LR). Hepatocytes were activated at 2h after PH, and their transition from G₀ phase to cell cycle occurred at 2-6h after PH (Guo et al., 2006; Xu et al., 2010). On the other hand, liver tissue was infiltrated with inflammatory cells, and necrosis occurred to some hepatocytes at 6h of rat acute hepatic failure (AHF) (Leu et al., 2003). Gene synergy value (E_t) of all paths of ERK1/2 signaling pathway were similar to those of the control at 6h of LR, indicating that cell proliferation-promoting activities occurred later in LR. E_t of path 4 which promoted cell proliferation at 6h of AHF was smaller than that of control, indicating that this path may inhibit cell proliferation, which is consistent with the actual physiological activities at this time point. It is inferred that ERK1/2 signaling pathway is involved in regulating cell proliferation of AHF at this time point, but not in that of LR.

It was shown that 6-12h after rat PH is G₁ phase of hepatocytes (Xu et al., 2010). Inflammatory response of liver was strengthened, liver lobule got damaged, and hepatocytes took on necrosis and apoptosis at 12h of AHF (Mookerjee et al., 2007). E_t of path 2, 8 and 9 which promoted cell proliferation and path 11 which inhibited cell proliferation were greater than that of control at 12h of LR, implying that path 2, 8 and 9 promote cell proliferation and path 11 inhibits cell proliferation at this time point. E_t of path 11 which inhibited cell proliferation was greater at 12h of AHF, suggesting that this path inhibit cell proliferation at this time point, which agrees with the actual physiological activities. It was concluded that ERK1/2 signaling pathway is involved in the regulation of cell proliferation of LR and AHF at this time point.

It was found that 12-24h after rat PH is S phase of hepatocytes (Xu et al., 2010). Immune and inflammatory responses of liver were strengthened, apoptosis of liver cells continued, and the liver injury got severely at 24h of AHF occurrence (Mookerjee et al., 2007). E_t of path 13 which inhibited cell proliferation was smaller than that of control at 24h of rat LR in this study, meaning that the cell proliferation-inhibiting effect of this path is

more weakened at this time point. E_t of all the paths were similar to those of the control at 24h of AHF, suggesting that cell proliferation does not start at this time point, which agrees with the actual physiological activities. The results above revealed that ERK1/2 signaling pathway is involved in cell proliferation of LR at this time point, but not in that of AHF.

Generally, 36-72h after rat PH is the second cell cycle of hepatocytes (Xu et al., 2010). Inflammatory response and cell apoptosis of liver were decreased, cell proliferation and differentiation of liver were increased, and liver function began to recover at 72h of AHF occurrence (Zhang et al., 2005; Pathikonda et al., 2010). E_t of all the paths were similar to those of the control at 72h of LR, implying that the paths above is not involved in cell proliferation of second cell cycle of hepatocytes at this time point. E_t of path 2 and 6 which promoted cell proliferation were greater, indicating that these two paths promote cell proliferation at this time point, which is in accordance with the actual physiological activities. It is obvious that ERK1/2 signaling pathway is involved in regulating cell proliferation of AHF at this time point, but not in that of LR.

In summary, Rat Genome 230 2.0 Array was used to detect the gene expression profiles of ERK1/2 signaling pathway in rat LR and rat AHF occurrence, and bioinformatics and systems biology methods to analyze the roles of fourteen paths of ERK1/2 signaling pathway in regulating rat LR and rat AHF occurrence in this study. It was found that seven paths of ERK1/2 signaling pathway participated in regulating cell proliferation and apoptosis of rat LR and rat AHF occurrence. The results above will be confirmed by using gene addition and gene interference *in vivo* and *in vitro* in the future.

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Figure 1

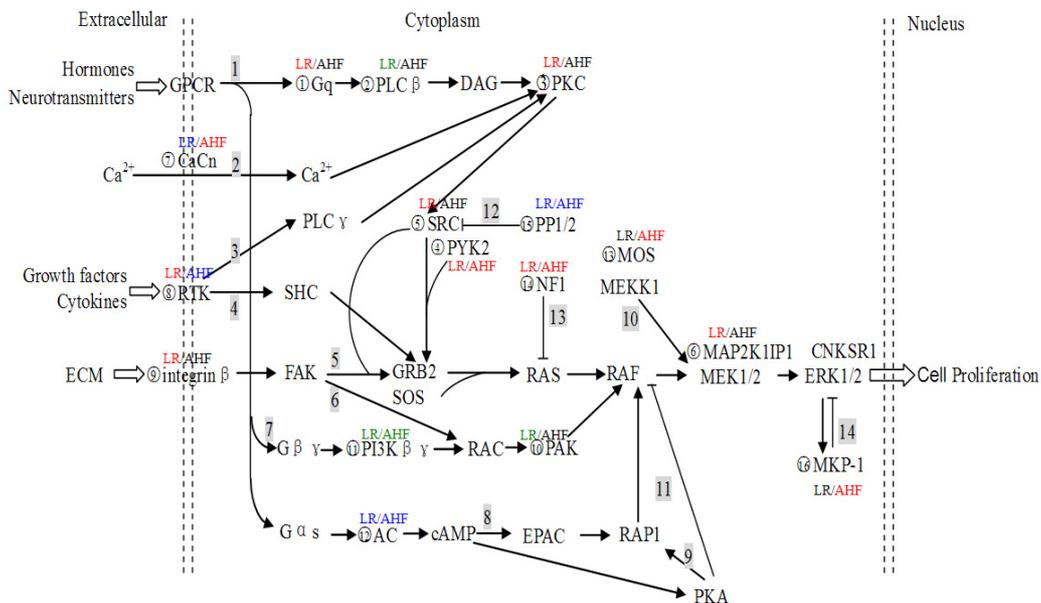


Figure 1. Fourteen path of ERK1/2 signaling pathway and genes related to these paths

Symbols in red mean the up-regulated genes, those in green the down-regulated, and those with yellow ground the up/down-regulated genes. The figures under grey ground present the ordinal numbers of the paths, and the figures in circle the ordinal numbers of proteins which share the same ordinal numbers in the table above.

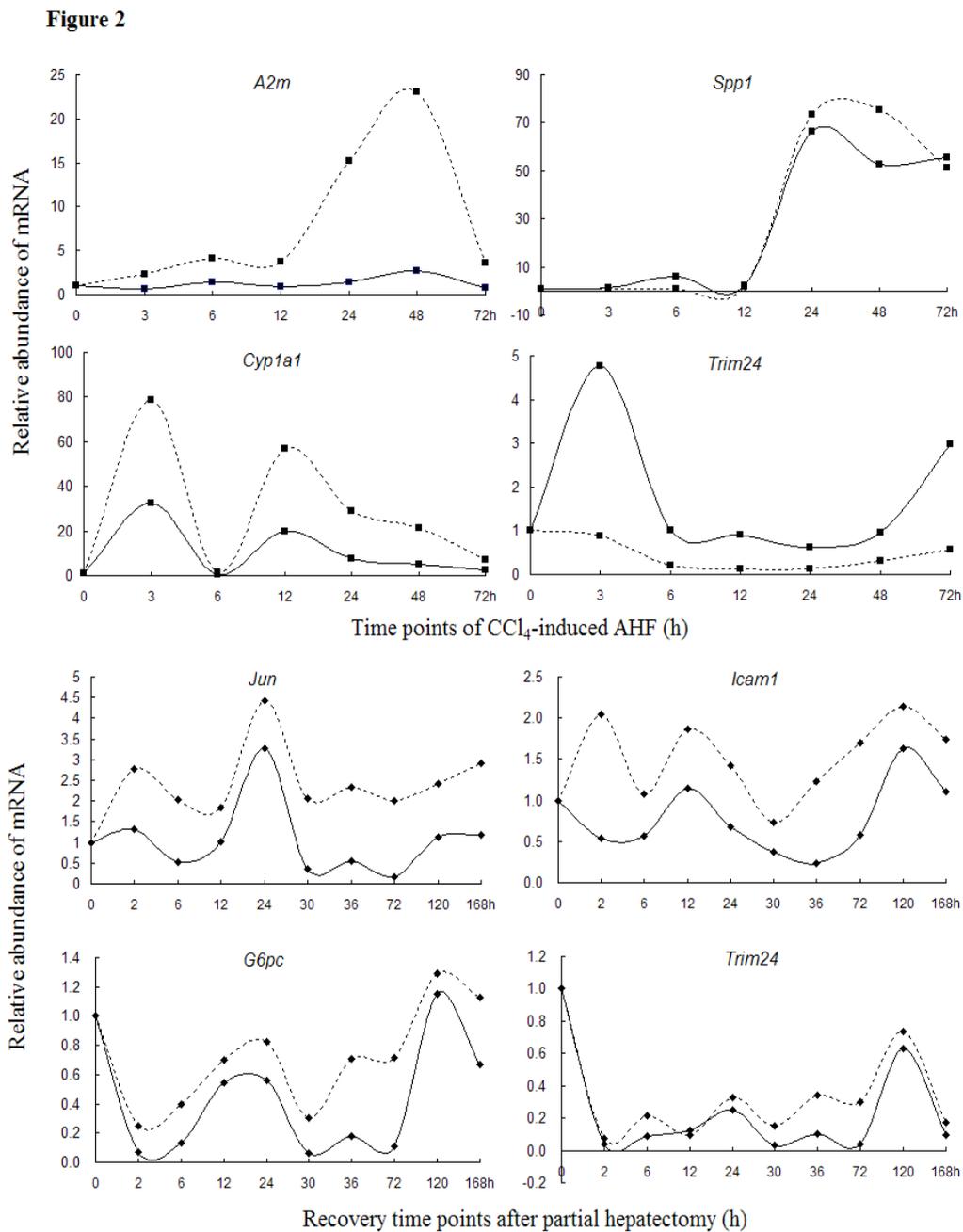


Figure 2. mRNA expression of four selected genes measured by microarrays and RT-PCR. Solid line presented the results of RT-PCR and dotted line that of Rat Genome 230 2.0 Array

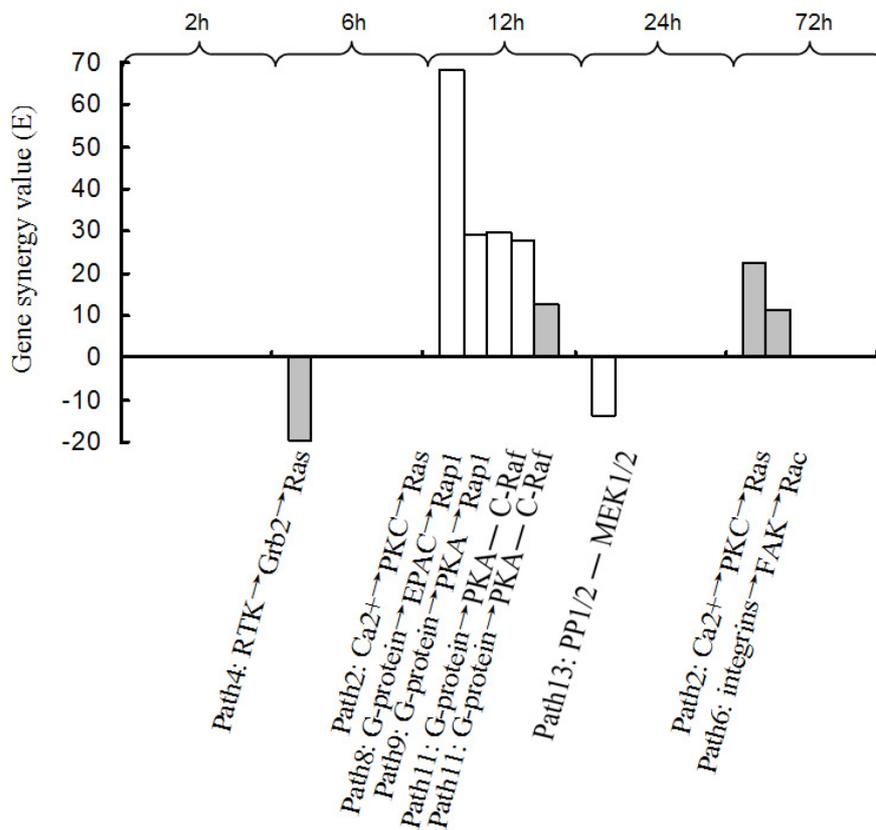


Figure 3. Cell proliferation regulated by thirty eight paths of ERK1/2 signaling pathway in rat liver regeneration (LR) and rat acute hepatic failure (AHF). X-axis: Path of ERK1/2 signaling pathway and time points of LR/AHF. □ LR-related path; ▨ AHF-related path