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Study on the Activity of PI3K/AKT, Death Receptor and 14-3-3 Mediated Signaling Pathways Regulating Hepatocyte Apoptosis during Rat Liver Regeneration

X.Q. Guo[1] --- *M.H. Li*[2] --- *T.T. Xu*[3] --- *C.F. Chang*[4] --- *G.P. Wang*[5] --- *C.S. Xu*[6*]

[1,2,3,4,5,6]College of Life Science, Henan Normal University, Xinxiang, China; Key Laboratory for Cell Differentiation Regulation, Xinxiang, China

Abstract

Studies have shown that apoptosis is closely related to the rat liver regeneration. To understand the mechanism of hepatocyte apoptosis during rat liver regeneration at the gene transcription level, the Rat Genome 230 2.0 Array was used to determine the expression changes of genes. Then the genes associated with cell apoptosis were searched by GO and NCBI databases, and cell apoptosis signaling pathways were searched by the database of QIAGEN and KEGG. Their signaling activities were calculated by spectral function E(t). The mechanism of hepatocyte apoptosis during rat liver regeneration was analyzed by Ingenuity Pathway Analysis 9.0 (IPA). The results showed that among the 27 signaling pathways regulating cell apoptosis, the E(t) values of Apoptosis signaling pathway and 14-3-3 mediated signaling pathway were significantly increased in the progression phase (6-72h after PH) of rat liver regeneration, and the E(t) values of hepatocyte apoptosis mediated by mitochondria rout were also significantly increased. The E(t) values of death receptor signaling pathway and PI3K/AKT branch of 14-3-3 mediated signaling pathway were significantly increased in the progression phase and the terminal phase (72-168h after PH) of rat liver regeneration, and the E(t) values of hepatocyte apoptosis mediated by cytomembrane route and nucleus route were also significantly increased. Conclusion: PI3K/AKT, death receptor and mitochondria branch played a key role in promoting cell apoptosis during rat liver regeneration.

Keywords: Rat liver regeneration, Signaling activity, Hepatocyte apoptosis, Ingenuity pathway analysis 9.0 (IPA), Rat genome 230 2.0 Array, Spectral function E(t).

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Contents

1. Introduction 94
2. Materials and Methods 94
2.1. Preparation of Rat Liver Regeneration Model and Hepatocyte Isolation and Identification 94
2.2. Rat Genome 230 2.0 Microarray Detection and Data Analysis 94
2.3. Real-Time Fluorescent Quantitative Polymerase Chain Reaction（qRT-PCR） 94
2.4. Confirmation of the Significant-Expressed Genes and Liver Regeneration-Related Genes in Rat Liver Regeneration....................95
2.5. Confirmation of Cell Apoptosis-Related Genes, the Cell Apoptosis Signaling Pathway- Associated Genes and Liver Regeneration-Involved Genes....................95
2.6. Calculation of Cell Apoptosis Activity Coefficient 95
2.7. Analysis of Gene Synergy 95
2.8. The Correlation Analysis of Rat Hepatocyte Apoptosis Activities with their Signaling Activities 95
2.9. Construction of the Interaction Network of Rat Hepatocyte Apoptosis Signaling Pathways- Related Genes...95
3. Results....................95
3.1. The Reliability Confirmation of The Rat Genome 230 2.0 Microarray Results 95
3.2. The Signaling Pathways Related with Hepatocyte Apoptosis and their Signaling Activity in Rat Liver Regeneration Indicated by the Changes in Gene Expression 96
3.3. The Expression Changes of the Genes Related to the Active Hepatocyte Apoptosis Signaling Pathways in Rat Liver Regeneration 96
3.4. The Expression Changes of the Hepatocyte Apoptosis-Related Genes During Rat Liver Regeneration 97

* Corresponding Author

3.5. The correlation of signaling activities of hepatocyte apoptosis signaling pathways with hepatocyte apoptosis activities during rat liver regeneration....98
3.6. The Regulation Role of Cell Apoptosis Signaling Pathways to Hepatocyte Apoptosis in Rat Liver Regeneration....98
4. Discussions 99
Reference....100

1. Introduction

Liver contains a variety of cell types including hepatocytes (HCs), biliary epithelial cells (BECs), oval cells (OCs) and so on [1]. Under normal physiological conditions, the majority of adult liver cells are in the quiet state [2, 3]. However, when after partial hepatectomy (PH), hepatocytes can rapidly enter cell cycles to compensate for the lost liver tissues and restore the function of the liver [4, 5]. Usually, the liver regeneration(LR) process was divided into three stages: priming phase (0.5-6h after PH), progression phase (6-72h after PH), and terminal phase (72-168h after PH), which involved many physiological activities, for example, cell activation, proliferation, re-differentiation and tissue structure reconstruction [6, 7], and the balance between cell apoptosis and cell proliferation is an important event during rat liver regeneration, which regulated by lots of factors and signaling pathways [8, 9].

Apoptosis is an active cell death process which is controlled by genes and is not only closely related to the body's normal development and tissue stability, but also related to many diseases, such as Parkinson's disease [10], cancer [11-13] and so on. Studies have shown that apoptosis can work through the cytomembrane, cytoplasm, mitochondria, endoplasmic reticulum, nucleus, etc. [14], including cytomembrane route, nucleus route, mitochondria route and endoplasmic reticulum route.

It is generally acknowledged that 27 signaling pathways regulate cell apoptosis, 26 of them involves in cytomembrane route, 23 of them involves in nucleus route, 12 of them involves in mitochondria route and 2 of them involves in endoplasmic reticulum route. Among them, apoptosis signaling and death receptor signaling involved in cytomembrane route, nucleus route and mitochondria route, they regulated cell apoptosis by activating transcription factor NF-κB and changing the mitochondrial outer membrane permeability to release apoptosis factor. 14-3-3 mediated signaling involved in cytomembrane route and nucleus route, the JNK, PI3K/AKT and Ras/ERK branches of it regulated cell apoptosis by activating transcription factors c-FOS, c-JUN, NF-κB and ELK-1. Studies showed that the above pathways and branches formed a complex networking, and their signaling type, quantity, time, patterns ect. need to study by principles and methods of the biological high throughput analysis and molecule interaction. Therefore, this article studied the role of signaling pathways in regulating liver cell differentiation at the gene transcription level.

2. Materials and Methods

2.1. Preparation of Rat Liver Regeneration Model and Hepatocyte Isolation and Identification

In the study, the adult male Sprague-Dawley rats (SD), weighing 230±20g, were obtained from animal center of Henan Normal University. The rats were housed in the room of 21±2 °C, relative humidity 60±10%， illumination 12h/d (8:00-20:00) with free access to water and food. A total of 114 rats were randomly divided into 19 groups with six rats per group: nine groups for two-thirds hepatectomy (PH), nine for sham operation (SO) and one control group. The rats in PH groups were subjected to two-thirds hepatectomy following the method of Higgins and Anderson [15]. After PH, the abdominal cavity was opened at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168h to collect their liver tissues. Then, the hepatocytes were isolated by two-step collagenase digestion and percoll density gradient centrifugation [16, 17], and identified by immunocytochemistry of their marker proteins ALB and G6P [18], and their viability was measured by MTT method Dudoit, et al. [19]. Purity and viability of the hepatocytes used in this study were over 95%. In the study, Animal Protection Law of China was obeyed.

2.2. Rat Genome 230 2.0 Microarray Detection and Data Analysis

In the study, total RNA was extracted and purified followed the protocols previously described [20]. The first chain of cDNA was synthesized by SuperScript II RT reverse transcription system, and the second was synthesized according to the guideline of Affymetrix cDNA kit. Biotin-labeled cRNA was prepared using GeneChip IVT kit as instructed by the manufacturer [21]. cRNA fragments of 35-200 bp were prepared by fragmentation reagent treatment. The Rat Genome 230 2.0 array was hybridized with the cRNA fragments, which were pretreated. Then, they were stained, washed automatically using GeneChip fluidics station 450 (Affymetrix Inc., Santa Clara, CA, USA), scanned using GeneChip scanner 3000 (Affymetrix Inc., Santa Clara, CA, USA), and the spots were converted into signal values using Affymetrix GCOS 2.0 software [22]. The signal values were normalized according to manufacturer's instruction. The p-values were determined using the probe signal. When the p-value of a gene is < 0.05, this gene is defined as present (P), < 0.065 is marginal (M), and > 0.065 is absent (A). In order to minimize the experimental operation and microarray test differences, each sample was repeated three times, and their average value was used to statistical analysis.

2.3. Real-Time Fluorescent Quantitative Polymerase Chain Reaction（qRT-PCR）

To validate the reliability of microarray data, the expression level of total 30 genes of hepatocytes including *THRB, PCK1* and *SIRPA* was examined by qRT-PCR. mRNA was prepared from the purified hepatocytes. Their primers were designed using Primer Express 5.0 software. Their first chain of cDNA was synthesized by SuperScript II RT reverse transcription system (Promega, USA). The PCR were performed by the conditions with Sybr Green I: 2min at 95°C, followed with 40 cycles for 15s at 95°C, 15s at 60°C, and 30s at 72°C. Each sample was performed in triplicates. β-ACTIN (NM_031144) was used as an internal control.

2.4. Confirmation of the Significant-Expressed Genes and Liver Regeneration-Related Genes in Rat Liver Regeneration

In this study, the genes with ratio values of PH/control and SO/control ≥3 or ≤0.33 were considered as significant expressed genes. The genes with F-test difference 0.01≤P<0.05 or P≤0.01 between PH and SO, and at least at one of PH time points in liver regeneration, were considered as liver regeneration-related genes.

2.5. Confirmation of Cell Apoptosis-Related Genes, the Cell Apoptosis Signaling Pathway-Associated Genes and Liver Regeneration-Involved Genes

To find out cell apoptosis-related genes, the term "cell apoptosis" was entered into website GO and NCBI. Then, the cell apoptosis-related genes were uploaded to "Canonical Pathway" of Ingenuity Pathway Analysis 9.0 (IPA) software, to obtain corresponding pathways. On the other hand, the term "cell apoptosis" was entered into website QIAGEN and KEGG, also to obtain corresponding pathways. Finally, the both shared pathways were selected for further analysis.

2.6. Calculation of Cell Apoptosis Activity Coefficient

In the study, ratio values of the cell apoptosis signaling pathway-related genes were uploaded to "Dataset Files" of IPA software, to calculate physiological activity coefficient –log (p-value) by "Comparison Analyses". The differences of –log (p-value) between PH and SO group were analyzed by F-test. When the differences of PH and SO were 0.01≤p≤0.05, meaning that the differences were significant, when p≤0.01, meaning very significant, both suggesting that the pathways regulated hepatocytes apoptosis in rat liver regeneration.

2.7. Analysis of Gene Synergy

In the study, the gene synergy was calculated according to spectrum function *E(t),* which was described by Xu, et al. [23]. In brief, the ratio values of cell apoptosis related-genes and cell apoptosis signaling pathways-related genes were calculated by spectrum function E(t), and the values in PH were compared with in SO. When the *E(t)* values in PH were greater than zero and SO, and the difference between PH and SO was significant, it indicated that the corresponding physiological activities were increased. When *E(t)* values in PH were less than zero and SO, and the difference between PH and SO was also significant, it meaned that the corresponding physiological activities were decreased. When there was no significant difference between *E(t)* values of PH and SO, , it showed that the physiological activities were similar between PH and SO.

2.8. The Correlation Analysis of Rat Hepatocyte Apoptosis Activities with their Signaling Activities

In the study, the ratio values of hepatocyte apoptosis signaling pathways-related genes were entered into computer to calculate the signaling activities *(E(t)* values) by spectrum function formula. On the other hand, the transcription factors activated by cell apoptosis signal pathways were input to TRED and Lymph TF-DB [24, 25] to obtain their downstream target genes. The cell apoptosis activities *(E(t)* values) were also calculated by that. Finally, the correlation of rat hepatocyte apoptosis signaling activities and cell apoptosis activities were confirmed by comparison.

2.9. Construction of the Interaction Network of Rat Hepatocyte Apoptosis Signaling Pathways-Related Genes

In the study, apoptosis signaling pathway, 14-3-3 mediated signaling pathway, Death Receptor signaling pathway were input respectively into "Pathways and tox lists" of IPA software to get the corresponding signaling pathways, branches, function, activated transcription factors, genes etc. Then, these signaling pathways were copied to "Pathway Designer" of the IPA software to obtain the signaling pathways' network.

3. Results

3.1. The Reliability Confirmation of The Rat Genome 230 2.0 Microarray Results

It is reported that the Rat Genome 230 2.0 Array contained 24,618 rat genes. It was used to detect gene expression abundance of hepatocytes at each time point of rat liver regeneration. The microarray results showed that the expression level of 9366 genes in hepatocytes, including 151 cell apoptosis signaling pathways-related genes and 1020 cell apoptosis-related genes, were significantly changed during rat liver regeneration, at least at one PH time point. To validate reliability of these results, 30 genes of them, such as *THRB, PCK1* and *SIRPA* were randomly selected for quantitative RT-PCR (qRT-PCR) analysis. The test results showed that both were generally consistent. Figure 1 provided the results of three genes, *THRB, PCK1* and *SIRPA* as detected by the two methods. The qRT-PCR results of other genes had previously published in the reference [26].

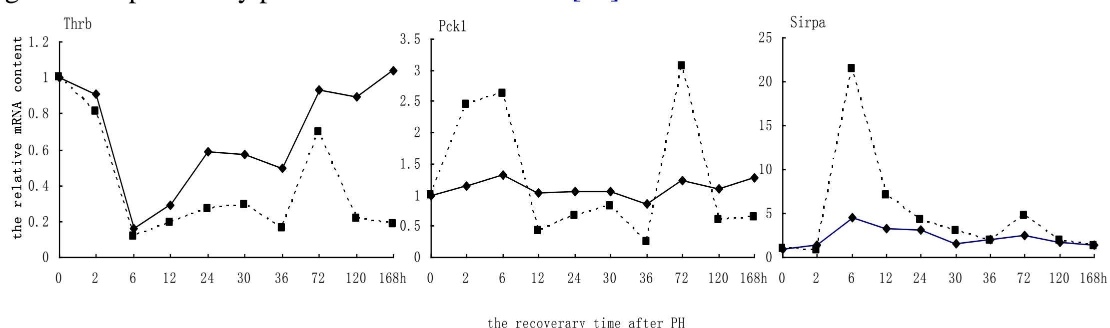

Figure-1. Comparison of mRNA levels detected by Affymetrix Rat Genome 230 2.0 arrays (solid lines) and real-time quantitative PCR (dotted lines).

3.2. The Signaling Pathways Related with Hepatocyte Apoptosis and their Signaling Activity in Rat Liver Regeneration Indicated by the Changes in Gene Expression

In the study, the values of gene expression abundance detected by Rat Genome 230 2.0 were input into IPA software to calculate the signaling activity coefficient of the signaling pathways, e.g. –log (p-value). F-test was used to analyze differences of the –log (p-value) between PH and SO group. It was found that 27 signaling pathways were related to cell apoptosis by analysis QIAGEN website and Canonical Pathways of Ingenuity Pathway Analysis 9.0 (IPA) software. Of them, the -log(p-value) of Apoptosis signaling pathway, 14-3-3 mediated signaling pathway and Death receptor signaling pathways in PH group were significantly different ($0.01 \le p \le 0.05$) as compared to SO group, the -log(p-value) of the rest signaling pathways were similar in PH and SO group (Table 1).

Table-1. The cell apoptosis signaling pathways of hepatocytes and their signaling activities during rat liver regeneration

Cell apoptosis related signaling pathways (CARSP)	Routes	Liver regeneration-related genes			The activity of
		UR	DR	UR/DR	
14-3-3-mediated signaling	AB	25	4	0	0.0282*
Apoptosis signaling	ABC	10	3	1	0.0471*
April mediated signaling	AB	11	0	0	0.4338
Calcium-induced T lymphocyte	ABD	9	0	0	0.5152
CD27 signaling in lymphocytes	ABC	9	1	0	0.8267
Ceramide signaling	ABC	20	4	0	0.3537
Cytotoxic T lymphocyte-mediated	AC	9	1	0	0.4979
Death receptor signaling	ABC	11	0	1	0.0419*
IL-1 signaling	AB	28	2	0	0.3686
IL-15 production	B	4	0	0	0.8909
IL-15 signaling	AB	11	3	0	0.4473
IL-3 signaling	AB	9	3	0	0.6953
IL-9 signaling	AB	8	2	0	0.4976
Induction of apoptosis by HIV1	ABC	12	0	1	0.2901
JAK/STAT signaling	AB	10	4	1	0.4042
LPS-stimulated MAPK signaling	AB	16	5	0	0.5252
Lymphotoxin β receptor signaling	AC	10	2	0	0.6615
Myc mediated apoptosis signaling	ABC	14	3	0	0.2267
Nur77 signaling in T lymphocytes	ABCD	9	0	0	0.7937
PTEN signaling	A	19	4	1	0.5023
Retinoic acid mediated apoptosis	AC	7	3	0	0.9756
SAPK/JNK signaling	AB	17	5	0	0.9206
Telomerase signaling	AB	19	6	0	0.2766
TNFR1 signaling	ABC	8	0	1	0.7344
TNFR2 signaling	AB	5	0	1	0.7448
Toll-like receptor signaling	AB	12	2	0	0.5463
TWEAK signaling	ABC	3	0	1	0.2901

*showing significant difference between PH and SO group ($P \le 0.05$), **indicating very significant difference between PH and SO group ($P \le 0.01$) ; A-D respectively stand for cytomembrane route, nucleus route, mitochondria route and endoplasmic reticulum route; UR stands for up-regulated; DR represents down-regulated; UR/DR shows up/down-regulated.

3.3. The Expression Changes of the Genes Related to the Active Hepatocyte Apoptosis Signaling Pathways in Rat Liver Regeneration

The gene expression abundances of hepatocyte apoptosis signaling pathways detected by Rat Genome 230 2.0 microarray revealed that 37 genes were up-regulated, 6 genes were down-regulated, 1 gene was up/down regulated in rat liver regeneration. Of them, apoptosis signaling pathway included NF-κB, mitochondria branches and 63 genes. 14 of them were rat liver regeneration-related, *TNF, PLCG2, PRKCQ* etc. 10 genes were up-regulated. *MRAS, GAS2* and *DFFB* etc. 3 genes were down-regulated. *XIAP* was up/down-regulated. 14-3-3 mediated signaling pathway included JNK, PI3K/AKT, Ras/ERK branches and 84 genes. 29 of them were rat liver regeneration-related, *TNF, SRC, PIK3R3* etc. 25 genes were up-regulated. *MRAS, PIK3C2G, PIK3R1* and *TUBA4A* etc. 3 genes were down-regulated. Transcription factors c-FOS and c-JUN in JNK branch were up-regulated. Death receptor signaling pathway included 44 genes. 12 of them were rat liver regeneration-related, *TNF, TNFRSF21, HSPB1* etc. 11 genes were up-regulated. XIAP was up/down-regulated (Table 2).

Table-2. The expression changes of genes related to cell apoptosis signaling pathways during rat liver regeneration

Genes	CAR SP	Routes	Recovery time (h) after partial hepotectomy (PH)								
			2	6	12	24	30	36	72	120	168
Akt3	2	A B	0.86	1.26	2.59	1.44	1.45	**6.80**	**4.42**	**3.66**	1.58
Bcl2	1 3	A B C	**3.07**	1.85	**4.39**	**3.60**	**5.06**	**4.98**	**6.04**	**3.06**	**3.15**
Bcl2l1	1	A B C	**3.46**	1.38	1.91	1.13	1.46	1.19	1.23	1.30	0.96
Bcl2l11	1	A B C	2.54	2.63	**3.70**	**3.13**	**3.05**	2.36	2.13	1.96	1.63
Cflar	3	A B C	2.07	**3.09**	2.27	1.85	1.09	1.08	1.80	1.17	1.38
Dffa	1	A B C	1.16	**3.49**	2.81	1.10	1.87	1.20	2.04	1.66	1.56
Dffb	1	A B C	0.87	1.25	1.31	1.31	0.65	1.42	0.86	0.92	**0.32**
Fas	1 3	A B C	1.84	0.66	1.26	**3.39**	**4.27**	2.07	**3.07**	**5.00**	**3.69**
Fos	2	A B	**5.02**	1.12	**4.73**	**14.96**	2.31	1.04	**5.21**	2.76	**4.23**
Gas2	1	A B C	0.68	**0.19**	**0.15**	0.58	0.79	0.73	0.70	0.76	0.91
Hspb1	3	A B C	**3.63**	**3.27**	1.04	**3.76**	**9.69**	**3.57**	**3.97**	**5.76**	**7.87**
Hspb2	3	A B C	1.11	1.95	**4.27**	2.01	**3.61**	2.22	2.20	**7.87**	2.92
Hspb3	3	A B C	**3.26**	2.33	1.95	2.57	**5.01**	2.72	2.04	1.57	0.87
Hspb7	3	A B C	2.74	**5.97**	2.49	**3.99**	**4.98**	**4.03**	**3.92**	**5.59**	**5.17**
Ikbkb	1 3	A B C	2.31	2.71	1.93	1.68	1.47	1.23	**3.21**	**3.04**	1.91
Jun	2	A B	**3.29**	2.68	1.97	**3.97**	1.54	1.91	2.14	1.75	2.18
Mapk1	2	A B	**3.52**	2.16	**3.99**	**3.02**	1.68	**3.73**	**4.66**	1.33	**6.31**
Mapk1	2	A B	1.09	2.19	1.40	2.82	0.59	**3.03**	**6.59**	2.26	2.44
Mapk8	1 2 3	A B C	2.77	1.28	2.34	1.66	2.04	1.32	**3.52**	1.56	2.27
Mapt	2	A B	2.16	**3.21**	2.66	2.93	**4.00**	**3.27**	**7.44**	**3.20**	**3.94**
Mras	1 2	A B C	**0.32**	0.88	0.76	0.71	0.66	0.50	0.80	0.66	0.63
Pik3c2	2	A B	0.80	0.77	**0.26**	**0.19**	0.35	0.33	0.38	0.76	0.92
Pik3r1	2	A B	1.34	0.92	0.77	0.59	0.85	0.69	**0.23**	0.39	0.54
Pik3r3	2	A B	2.31	1.17	**3.21**	1.38	2.33	2.61	2.61	1.18	2.81
Pik3r6	2	A B	1.22	1.66	1.31	1.55	1.73	1.21	**7.61**	1.52	1.51
Plcd4	2	A B	2.02	2.10	**8.38**	1.09	1.11	1.13	**4.21**	1.54	1.42
Plcg2	1 2	A B C	1.16	1.88	0.93	0.90	1.24	0.89	**3.27**	0.83	0.97
Plcl1	2	A B	1.13	0.37	0.57	0.80	0.69	**4.86**	1.50	0.83	0.60
Prkch	2	A B	1.38	0.94	1.40	**3.54**	1.00	2.41	**4.45**	1.86	**4.21**
Prkcq	1 2	A B C	**3.14**	1.07	**6.94**	**4.63**	**5.33**	**3.36**	1.81	**3.30**	0.98
Prkcz	2	A B	**4.11**	0.71	2.78	2.88	2.42	0.70	2.43	**6.83**	1.70
Sfn	2	A B	2.17	**3.31**	2.14	2.29	1.73	1.16	**4.04**	2.32	2.63
Src	2	A B	1.33	1.77	2.40	2.06	**3.11**	1.89	**4.78**	2.04	1.48
Stk11	2	A B	2.60	2.75	1.51	2.74	1.30	2.48	**3.16**	1.72	1.98
Tnf	1 2 3	A B C	1.71	**4.98**	2.26	1.96	2.80	**3.65**	**3.40**	2.03	1.39
Tnfrsf2	3	A B C	**29.29**	**64.65**	**108.8**	**32.68**	**7.99**	**14.55**	**14.73**	**7.44**	**4.09**
Tsc1	2	A B	2.25	1.71	**3.53**	**3.73**	2.69	1.53	2.49	2.52	**3.73**
Tuba1a	2	A B	1.81	0.68	1.63	2.62	**3.46**	**3.29**	0.88	0.83	0.67
Tuba4a	2	A B	0.94	0.42	1.34	1.38	1.24	1.10	**0.32**	0.49	0.86
Tuba8	2	A B	2.38	1.38	**5.90**	1.40	**4.05**	**3.36**	1.51	1.74	2.06
Tubb6	2	A B	**23.13**	**12.76**	**39.60**	**37.44**	**54.65**	**46.57**	**14.61**	**7.84**	**4.76**
Xiap	1 3	A B C	0.99	0.56	1.63	0.98	**3.81**	1.03	0.43	**0.31**	1.06
Ywhah	2	A B	1.34	1.48	2.13	2.08	**3.09**	2.49	1.05	1.28	1.05

PH stands for partial hepatectomy; LR stands for liver regeneration; A-C respectively stand for cytomembrane route, nucleus route and mitochondria route; UR stands for up-regulated; DR represents down-regulated; UR/DR shows up/down-regulated

3.4. The Expression Changes of the Hepatocyte Apoptosis-*R*elated Genes During Rat Liver Regeneration

It was found that 3903 genes were cell apoptosis-related by checking GO and NCBI library, and that Rat Genome 230 2.0 contained 2805 of them with 1020 genes related to liver regeneration. Of the 1020 genes, A2M, BDNF, CCNA1 etc. 857 genes were up-regulated, ABCB9, BMF, EGF etc. 140 genes were down-regulated, BLM, CCNA2, ERG etc. 23 genes were up/down-regulated. Apoptosis signaling pathway regulated 67 genes related to hepatocyte apoptosis. Of the 67 genes, 60 genes were up-regulated, 7 genes were down-regulated. 14-3-3 mediated signaling pathway regulated 89 genes related to hepatocyte apoptosis. Of the 89 genes, 82 genes were up-regulated, 7 genes were down-regulated. Death receptor signaling pathway regulated 67 genes related to hepatocyte apoptosis. Of the 67 genes, 60 genes were up-regulated, 7 genes were down-regulated (Table 3, Schedule 3).

Table-3. The expression changes of hepatocyte apoptosis-related genes during the rat liver regeneration

Pathways and TFs	Rou tes	Recovery time (h) after partial hepotectomy (PH)									
		2	6	12	24	30	36	72	120	168	Total
		U/D	U/D	U/D	U/D	U/D	U/D	U/D	U/D	U/D	U/DR/UDR
1	AB	24/0	20/1	22/2	28/2	26/1	25/1	40/1	16/0	15/0	60/7/0
2	AB	35/0	27/1	31/2	41/2	31/1	32/1	52/1	22/0	22/0	82/7/0
3	AB	24/0	20/1	22/2	28/2	26/1	25/1	40/1	16/0	15/0	60/7/0
1 2 3 → NF-	AB	24/0	20/1	22/2	28/2	26/1	25/1	40/1	16/0	15/0	60/7/0
2 → ELK-	AB	6/0	6/0	5/0	7/0	3/0	5/0	5/0	2/0	4/0	9/0/0
2 → c-FOS	AB	5/0	5/0	5/0	4/0	3/0	2/1	6/0	1/0	2/0	8/1/0
2 → c-JUN	AB	14/0	10/0	10/0	15/0	9/0	11/1	21/1	7/0	9/0	33/2/0
Total		35/0	27/1	31/2	41/2	31/1	32/1	52/1	22/0	22/0	82/7/0

PH stands for partial hepatectomy; TFs stand for transcription factors;1-3 respectively mean Apoptosis signaling pathway, 14-3-3 mediated signaling pathway and Death receptor signaling pathway; A-C respectively stand for cytomembrane route, nucleus route, and mitochondria route; UR stands for up-regulated; DR represents down-regulated; UR/DR shows up/down-regulated.

3.5. The correlation of signaling activities of hepatocyte apoptosis signaling pathways with hepatocyte apoptosis activities during rat liver regeneration

In this paper, spectrum function *(E(t))* was used to analyze the correlation of the signaling activities with hepatocyte apoptosis activities. The results showed that signaling activity *(E (t)* values) of Apoptosis signaling pathway was higher than those of SO groups at 2h, 12h and 30-36h after PH, and the hepatocyte apoptosis activity *(E (t)* values) was higher than those of SO groups at 24-120h after PH. The signaling activity *(E (t)* values) of its NF-κB branch had no significant difference with SO, and the hepatocyte apoptosis activity *(E (t)* values) was higher than those of SO groups at 24-120h after PH. The signaling activity *(E (t)* values) of its mitochondria branch was higher than those of SO groups at 12h and 30h after PH.

The signaling activity *(E (t)* values) of 14-3-3 mediated signaling pathway was higher than those of SO groups at 2-72h after PH, and the hepatocyte apoptosis activity *(E (t)* values) was higher than those of SO groups at 24-120h after PH. The signaling activity *(E(t)* values) of its JNK branch had no significant difference with SO, and the hepatocyte apoptosis activity *(E(t)* values) was higher than those of SO groups at 24h and 36-120h after PH. The signaling activity *(E(t)* values) of its PI3K/AKT branch was higher than those of SO groups at 2h, 12h and 30-72h after PH, and the hepatocyte apoptosis activity *(E(t)* values) was higher than those of SO groups at 24-120h after PH. The signaling activity *(E(t)* values) of its Ras/ERK branch had no significant difference with SO, and the hepatocyte apoptosis activity *(E(t)* values) was higher than those of SO groups at 12-72h after PH.

The signaling activity *(E(t)* values) of Death receptor signaling pathway was higher than those of SO groups at 2-168h after PH, and the hepatocyte apoptosis activity *(E(t)* values) was higher than those of SO groups at 24-120h after PH. The signaling activity *(E(t)* values) of its NF-κB branch had no significant difference with SO, and the hepatocyte apoptosis activity *(E(t)* values) was higher than those of SO groups at 24-120h after PH. The signaling activity *(E(t)* values) of its mitochondria branch was higher than those of SO groups at 12h and 30h after PH (Table 4).

Table-4. The correlation of signaling activities of Apoptosis signaling pathway, 14-3-3 mediated signaling pathway and Death receptor signaling pathways with hepatocyte apoptosis activities during rat liver regeneration.

Pathways and branches(E(t))	Rout es	Recovery time (h) after partial hepotectomy (PH)								
		2	6	12	24	30	36	72	120	168
		a/b	a/b	a/b	a/b	a/b	a/b	a/b	a/b	a/b
Apoptosis signaling	ABC	+/~	~/~	+/~	~/+	**+/+**	**+/+**	~/	~/+	+/~
NF-κB branch	AB	~/~	~/~	~/~	~/+	~/+	~/+	~/	~/+	~/~
Mitochondria branch	C	~/—	~/—	**+/—**	~/—	**+/—**	~/—	~/—	~/—	~/—
14-3-3 mediated signaling	AB	+/~	+/~	+/~	**+/+**	**+/+**	**+/+**	**+/+**	~/+	~/~
JNK branch	AB	~/~	~/~	~/~	~/+	~/~	~/+	~/+	~/+	~/~
sPI3K/AKT branch	AB	+/~	~/~	+/~	~/+	**+/+**	**+/+**	**+/+**	~/+	~/~
Ras/ERK branch	AB	~/~	~/~	~/+	~/+	~/+	~/+	~/+	~/~	~/~
Death receptor signaling	ABC	+/~	+/~	+/~	+/+	+/+	+/+	+/+	+/+	+/~
NF-κB branch	AB	~/~	~/~	~/~	~/+	~/+	~/+	~/+	~/+	~/~
Mitochondria branch	C	~/	~/—	**+/—**	~/—	**+/—**	~/—	~/—	~/—	~/—

PH stands for partial hepatectomy; A-C respectively stand for cytomembrane route, nucleus route, and mitochondria route; "a" stands for signaling activities; "b" stands for hepatocyte survival activities. "+" represents PH group was significantly stronger than the control ; "~" stands for no significant difference between PH and the control.

3.6. The Regulation Role of Cell Apoptosis Signaling Pathways to Hepatocyte Apoptosis in Rat Liver Regeneration

The results showed that the ways and patterns which Apoptosis signaling pathway, 14-3-3 mediated signaling pathway and Death receptor signaling pathway regulated hepatocyte apoptosis were delineated as following.

Apoptosis signaling pathway was involved in the branches of TNF/FasL→TNFR/Fas→NIK→IKK →IκB/ NF-κB→ NF-κB, TNF/FasL→TNFR/Fas→Caspase8/10→ Bid→ BAX/BAK→ Cytochrome C/APAF1→Caspase 9→Caspase 3/7→Apoptosis,TNF/FasL→TNFR/Fas→ASK→MKK4/7→JNK1→BCL2→AIF/Endo G→Apoptosis, TNF/FasL→ TNFR/Fas→Ras→c-Raf→MEK1/2→ERK1/2→p90RSK→BAD→BCL-XL→ Apoptosis.14-3-3 mediated signaling pathway was involved in the branches of TNF-α→TNFR1→TRAF2→ ASK1→JNK→c-FOS /c-JUN, RTK→ PI3K→AKT→NF-κB/BAD-(14-3-3),RTK→GRB2→RAS-(14-3-3)→cRAF→MEK1/2-(14-3-3)→ERK1/2→ ELK1. Death receptor signaling pathway was involved in the branches of TNF/FasL→TNFR/Fas/DR3/4/5/6/→DAXX/ FADD/TRADD→Caspase8/10→Bid→BAX/BAK→ Cytochrome C/APAF1→ Caspase 9→Caspase 3/7→Apoptosis, TNF/FasL→TNFR/Fas/DR3/4/5/6/→DAXX/FADD/TRADD→ASK→MKK4/7→JNK1→BCL2→AIF/EndoG→ Apoptosis, TNF/FasL→ TNFR/Fas/DR3/4/5/6/→ DAXX/FADD/TRADD→ Ras→ c-Raf→ MEK1/2→ ERK1/2→ p90RSK→ BAD→ BCL-XL→Apoptosis (Figure 2).

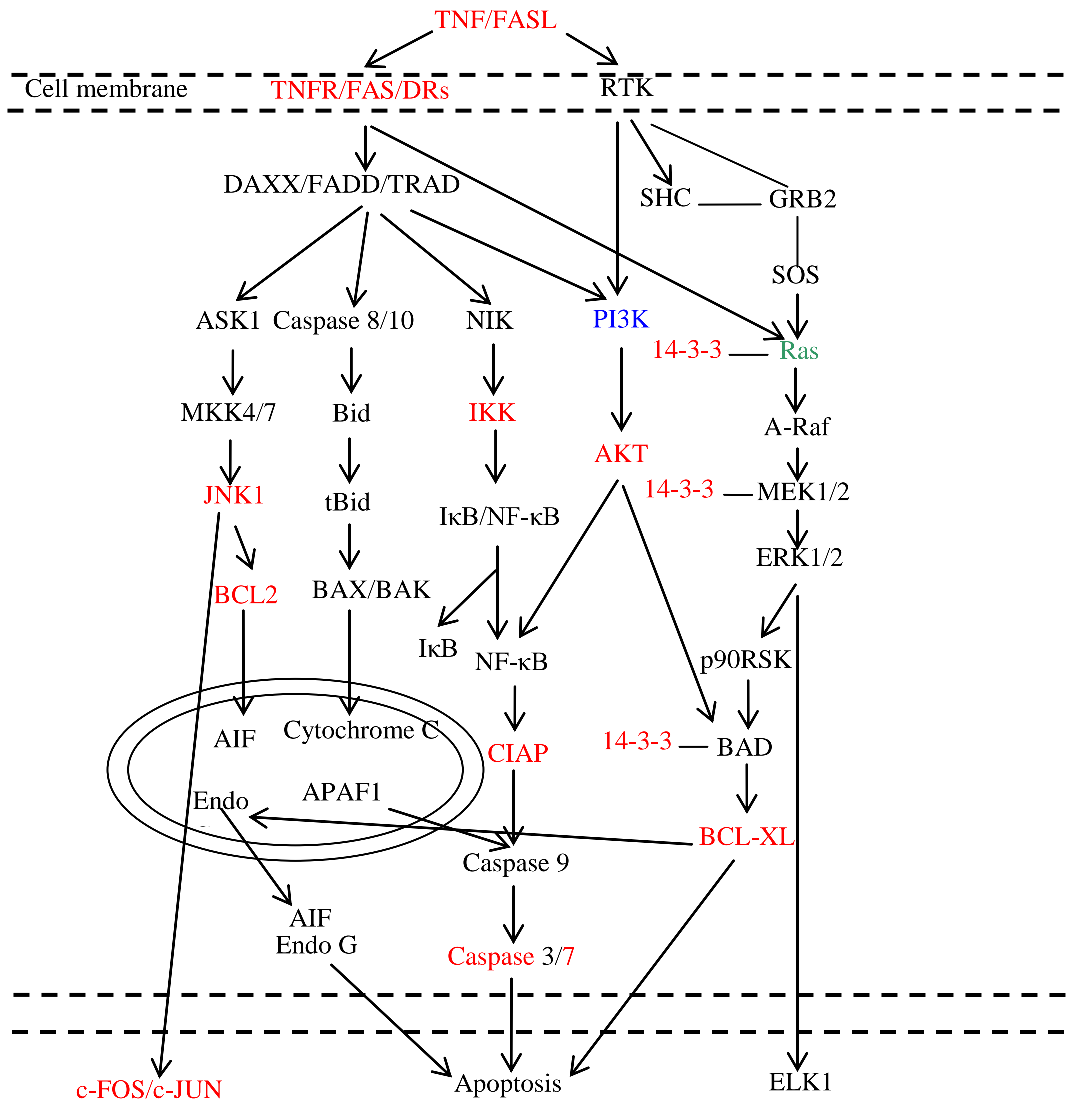

Figure-2. The regulation ways and patterns of signaling pathways to hepatocyte apoptosis in rat liver regeneration.

Red stands for the up-regulation genes; green for down-regulation; blue for up/down-regulation; black for no significant expression changes.

4. Discussions

Liver regeneration involves a sequence of orchestrated events including activation/expression of a series of cytokines and growth factors, accompanied by cell proliferation, cell differentiation and dedifferentiation, and tissue structure reconstruction, etc. Studies have shown that Fas is a typical death receptor, and plays an important role in mediating the process of apoptosis [27]. After the activation of Fas by its ligand FasL, the adapter protein FADD (Fas associated death domain) is raised, which in turn raises pro-caspase8/10 to form Fas-FasL-FADD-pro-caspase8/10, called death-inducing signaling complex (death-inducing signaling complex, DISC), then pro-caspase8/10 become active catalytic form by cracking itself. In liver cells, only a small amount of caspase8/10 is activated, thus it needs to amplify the apoptosis signal by mitochondrial rout. Activated caspase8/10 can not only cut proapoptotic molecule Bid, a member of Bcl-2 family, becomes tBid (truncated Bid), further activates the apoptosis-promoting factor BAX and BAK, and also can translocate to the mitochondria to induce the release of cytochrome c. The release of cytochrome c induces the formation of apoptotic complex, thereby activating pro-caspase9, and cutting performer Caspase 3 and Caspase 7 plasminogen to produce active Caspase 3 and Caspase 7 [28]. In addition, Fas can also interact with Daxx [29]. The activated Daxx, in turn, associates with and activates the apoptosis signal-regulating kinase 1 (ASK1) to induce the JNK signaling pathways [30]. The persistent activation of the JNK signaling pathways is thought to cause apoptotic cell death [31]. In this study, FAS encoding death receptor FAS was up-regulated at 24h, 30h and 72-168h after PH, *TNFRSF21* encoding death receptor DR6 was up-regulated at 2-168h after PH, *MAPK8* encoding JNK was up-regulated at 72h after PH, *FOS* encoding transcription factor c-FOS was up-

regulated at 2-24h,72-168h after PH, *JUN* encoding transcription factor c-JUN was up-regulated at 2h and 24h after PH. The signaling activities *(E(t)* values) of apoptosis signaling pathway, 14-3-3 mediated signaling pathway and their mitochondria branch, and the *E(t)* values of hepatocyte apoptosis activity were significantly higher at progression phase (6-72h after PH) than those of SO groups. The signaling activities *(E(t)* values) of death receptor signaling pathway and the *E(t)* values of hepatocyte apoptosis activity were significantly higher at progression phase and terminal phase (72-168h after PH) than those of SO groups. This indicated that apoptosis signaling pathway, 14-3-3 mediated signaling pathway and their mitochondria branch promoted hepatocyte apoptosis in the progression phase of rat liver regeneration. Death receptor signaling pathway promoted hepatocyte apoptosis in the progression and terminal phase of rat liver regeneration.

TNF has been shown to induce the caspase cascade by binding and activating their membrane receptor TNF receptor-1 (TNFR1), TNFR1 associates with the TNFR-associated death domain protein (TRADD) through the death domain-death domain interaction in the cytoplasmic side. TNF receptor-associated factor 2 (TRAF 2), which binds to the NH2-terminal region of TRADD, is essential for protective signals, and leads to nuclear translocation of NF-κB in hepatocytes. NF-κB is retained in an inactive form in the cytoplasm through association with one of the IκB inhibitory proteins. After cellular stimulation, the phosphorylation, ubiquination, and subsequent proteolysis of IκBα proteosomes enables NF-κB to translocate into the nucleus, where it regulates the transcription of NF-κB-responsive genes by interacting with κB binding sites. An important feature of NF-κB is to activate the gene transcription of anti-apoptosis, which inhibits cell apoptosis [32]. Moreover, cell survival and growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) can activate NF-κB by serine/threonine kinase AKT (namely PI3K/AKT pathway), which inhibits cell apoptosis through activating the transcription of the inhibitor of apoptosis [33, 34]. In this study, *TNF* was up-regulated at 6h, 36-72h after PH, *PRKCZ*, *SFN* and *YWHAH* encoding 14-3-3 was respectively up-regulated at 2 and 120h, 6 and 72h, 30h after PH, *AKT3* encoding AKT was up-regulated at 36-120h after PH, *XIAP* encoding inhibitor of apoptosis was up-regulated at 30h and down-regulated at 120h after PH. The signaling activities *(E(t)* values) of PI3K/AKT branch in 14-3-3 mediated signaling pathway, and the *E(t)* values of hepatocyte apoptosis activity were significantly higher at progression phase and terminal phase (72-168h after PH) than those of SO groups. This indicated that PI3K/AKT branch inhibit hepatocyte apoptosis in the progression phase and promoted hepatocyte apoptosis at terminal phase of rat liver regeneration.

In summary, this study found that apoptosis signaling pathway, death receptor signaling pathway and 14-3-3 mediated signaling pathway were positive correlattion with hepatocyte apoptosis activity at genomics level. we also studied the correlattion of these signaling pathways with hepatocyte apoptosis activity at proteomics level, and found the research conclusion at genomics level and proteomics level were comparable (manuscript is in preparation). In the future, we will further study their other roles by gene knockout, over expression, RNA interference ect.

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