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ORIGINAL ARTICLE

Comparative Analysis of Expression Profiles of Reg Signaling Pathways-Related Genes Between AHF and HCC

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Abstract

Regenerating islet-derived protein (Reg) could participate in the occurrence of diabetes mellitus, inflammation, tumors, and other diseased or damaged tissues. However, the correlation of Reg with acute hepatic failure (AHF) and hepatocellular carcinoma (HCC) is poorly defined. To reveal the expression profiles of Reg family and their possible regulatory roles in AHF and HCC, rat models of HCC and AHF were separately established, and Rat Genome 230 2.0 was used to detect expression profiles of Reg-mediated signaling pathways-associated genes from liver tissues in AHF and HCC. The results showed that a total of 79 genes were significantly changed. Among these genes, 67 genes were the AHF-specific genes, 45 genes were the HCC-specific genes, and 33 genes were the common genes. Then, K-means clustering classified these genes into 4 clusters based on the gene expression similarity, and DAVID analysis showed that the above altered genes were mainly associated with stress response, inflammatory response, and cell cycle regulation. Thereafter, IPA software was used to analyze potential effects of these genes, and the predicted results suggested that the Reg-mediated JAK/STAT, NF-κB, MAPK (ERK1/2, P38 and JNK), PLC, and PI3K/AKT signaling pathways may account for the activated inflammation and cell proliferation, and the attenuated apoptosis and cell death during the occurrence of AHF and HCC.

Keywords Regenerating islet-derived protein · Acute hepatic failure · Hepatocellular carcinoma · Gene expression profile · Physiological activities

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Introduction

Regenerating islet-derived protein (Reg), also known as hepatocarcinoma-intestine-pancreas (HIP) or pancreatitis-associated protein (PAP), is a secreted phosphorylated protein which belongs to C-type lectin superfamily. It has been found that Reg is expressed in diabetes mellitus, inflammation, tumors, and other diseased or damaged tissues, but is not expressed in normal pancreas and pancreatic juice. To date, 17 Reg family members have been found in human, rat, mouse, and varieties of other mammals, and they have a similar genetic structure. In pathological processes, PAP and its isoforms have been mainly investigated regarding responses to inflammation and stress (Jin et al. 2011).

Moreover, as the largest digestive gland in body, liver has been paid much attention and its relationships with the Reg family has also caused great attention of researchers. Christa et al. have found that HIP/PAP serum levels were increased in 21/28 (75%) patients with hepatocellular carcinoma (Christa et al. 1999). Cervello et al. have pointed out that expression level of HIP/PAP transcripts played multifarious roles in the occurrence of hepatoma (Cervello et al. 2002). HIP/PAP has been reported to be overexpressed in hepatocellular carcinoma (HCC) and contributes to the migration and invasion of HCC cells (Wang et al. 2015). A previous research has demonstrated that the upregulation of REG3A and REG1A expression was significantly correlated to HCC and hepatoblastomas (Cavard et al. 2006). Moreover, the deletion of Reg has been confirmed to enhance the sensitivity of liver to Fas-induced oxidative stress in acute liver failure model (Moniaux et al. 2011). Additionally, a study of Reg3α has shown that it could alleviate acute liver failure through its free-radical scavenging activity (Moniaux et al. 2015). All the above studies demonstrated that acute hepatic failure (AHF) and HCC are closely related to Reg family.

Recent studies suggested that Reg family was involved in the occurrence of squamous esophageal cancer, gastric endocrine carcinoma, and neural stem cells by the JAK/STAT, NF-κB, ERK1/2, P38, JNK, Phospholipase C (PLC), and PI3K/AKT signaling pathways (Eyre and Baune 2012; Wakita et al. 2015; Yamauchi et al. 2015). To further compare gene expression profiles of the Reg-related signaling pathways between AHF and HCC, this study separately established models of rat AHF and HCC induced by CCl_4 and diethylnitrosamine (DENA), and employed systems biology and ingenuity pathway analysis (IPA) software to predict the possible roles of Reg-related pathways in AHF and HCC, which would be helpful to reveal the relationships between the expression changes of the Reg-related signaling pathways and the predicted biological processes in AHF and HCC.

Methods and Materials

Animals

Health adult Sprague–Dawley rats, each of which weighed 190–210 g, supplied by the Experimental Animal Center of Henan Normal University, were housed in standard controlled room (21 ± 2 °C) with a 12:12-h light–dark cycle whose lights period was 8:00–20:00. All rats were fed a standard rodent chow diet with free access to distilled water, and received humane care in accordance with institutional guidelines. The procedures for care and use of animals, which were approved by the Ethics Committee of the Henan Normal University, were based on treating animals, preventing or reducing dynamic stress, pain and injury, respecting animal life, stopping the principle of barbaric behavior against animals, and handling animals with the least pain.

Preparation of Rat Models of AHF and HCC

AHFModel

Carbon tetrachloride (CCl4) was widely used to generate an experimental model mimicking acute liver failure caused by toxic substances. A total of 28 male rats were randomly divided into 7 groups including 6 model groups and one control group ($n = 4$). CCl_4 was diluted with sesame oil (2:3, v/v) under the sterile conditions. The rats in AHF group were fed with a single dose of 4 ml/kg diluted CCl_4 (Mikami et al. 2005), and liver right lobes were chosen for use at each of the following time points: 3, 6, 12, 24, 48, and 72 h.

HCC Model

A total of 36 male rats were randomly separated into six groups which contained 5 model groups and one control group ($n = 6$). Rats in the DENA group underwent intragastric administration of DENA (7 mg/100 g) according to the method which was stated in our previous research (Xu et al. 2011). Right lobes of liver were chosen for use at 5, 8, 12, 16, and 18 weeks after DENA treatment, respectively.

Histopathological Detection of Liver Tissue Changes in AHF and HCC

To verify histopathological changes of liver tissues in AHF and HCC, histopathological examinations were performed according to our laboratory methods (Xu et al. 2011; Wang et al. 2016). Briefly, small cuboids of about 5 × 5 mm × (2–3) mm from the right lobe of the liver were fixed and washed. Then they were routinely

dehydrated, cleared, and embedded in paraffin. Afterwards, the slices were stained with hematoxylin and eosin (HE), and finally were dehydrated, cleared, and sealed.

Rat Genome 230 2.0 Microarray Detection

For the two above-mentioned models of AHF and HCC, 100–200 mg tissues from the middle part of liver right lobe were taken, and total RNA from each sample was isolated from the liver tissues according to the manual of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then purified following the RNeasy mini protocol (Qiagen, Valencia, CA, USA). The quality of total RNA samples was assessed by optical density measurement at 260/280 nm and agarose electrophoresis with the ratio of 28S rRNA to 18S rRNA intensities. Then transcription profiles of liver tissues were determined by Rat Genome 230 2.0 chips as previously stated, and the experimental procedure mainly included cDNA synthesis, in vitro transcription, cRNA hybridization, microarray wash, image scanning, and data analysis (Xu et al. 2008a; Xu et al. 2008b).

Identification of AHF- and HCC-Related Genes

The images showing gene expression abundance were converted into signal values, and the signal values of each array were normalized as previously described (Xu et al. 2013). When ratio value was more than or equal to 2, it showed that gene expression was significantly up-regulated. When ratio value was less than or equal to 0.5, the result showed that gene expression was significantly down-regulated. When ratio value was 0.5–2, the data suggested that gene expression was biologically non-significant. The significantly expressed genes, whose values were the averages of three independently repeated measurements, were considered as rat AHF- and HCC-related genes.

Real-Time RT-qPCR

To verify the chip data, the primers used in this study were designed using Primer Express 2.0 software according to mRNA sequences of five target genes including *Myc*, *Ccnd1*, *Ccl2*, *Spc24*, and *Alpl* and internal control *β-actin*, and synthesized by Shanghai Generay Biotech Ltd (Table 1). Liver tissues were obtained from the near middle part of liver right lobe of each rat, and total RNA was extracted according to the manual of Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (2μg) was reverse-transcribed using random primers and Reverse Transcription kit (Promega, Madison, USA), and first-strand cDNA samples were subjected to quantitative PCR by using SYBR$^{@}$ Green I on Rotor-Gene 3000A (Corbett Robotics, Brisbane, Australia). As described previously, RT-qPCR was carried out by using above-mentioned specific primers, and the relative expression changes of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Wang and Xu 2010).

Table 1 Primer sequences used in real-time quantitative RT-PCR

Genes	Accession numbers	Primer sequences	Amplified products
Myc	NM_012603	FP : 5′-GAGGAGAAACGAGCTGAAGCG-3′	126 bp
		RP:5′-TGAACGGACAGGATGTAGGC-3′	
Ccl2	NM_031530	FP : 5′-AATGAGTCGGCTGGAGAA-3′	280 bp
		RP : 5′-GCTTGAGGTGGTTGTGGA-3′	
Ccnd1	NM_171992	FP : 5′-CCTGACTGCCGAGAAGTTGTGC-3′	251 bp
		RP : 5′-TGGAGGGTGGGTTGGAAATGAA-3′	
Alpl	NM_013059	FP : 5′-CATCGGACCCTGCCTTACCA-3′	231 bp
		RP : 5′-CGTGTCTCCTCGCCCGTGTT-3′	
Spc24	NM_026282	FP : 5′-AGAAGTTCATCAGCGACTACCT′-3′	185 bp
		RP : 5′-GGCCTGGACTCAACGAGA-3′	
β-actin	NM_031144	FP : 5′-CATCCGTAAAGACCTCTATGCCAACA-3′	109 bp
		RP : 5′-GTGCTAGGAGCCAGGGCAGTAATCT-3′	

Bioinformatics Analysis

In order to elucidate the similarities and differences of expression patterns of Reg signaling pathways-related genes in rat AHF and HCC, the above-mentioned genes were classified by K-means clustering, and then the genes in each cluster were used for DAVID analysis. DAVID bioinformatics resource includes an integrated biological knowledgebase and analytic tools with the purpose of systematically extracting biological meaning from large gene lists. Firstly, genes in each cluster were uploaded, and the module of functional annotation chart was used to extract and summarize annotation categories associated with group of genes (Huang et al. 2009). Then, Expression Analysis Systematic Explorer (EASE) as a network platform was used to analyze the functions of differentially expressed genes. The most important feature of EASE is to determine whether the Gene Ontology (GO) categories are over-represented or not according to a modified Fisher's exact test (Otu et al. 2007). According to EASE score (P value), the functionalcategories with EASE scores of < 0.05 were considered to be significantly over-represented during the occurrence and development of AHF and HCC.

Ingenuity Network Analysis (IPA)

In order to predict the regulatory roles of Reg signaling pathways-related genes in AHF and HCC, differentially expressed genes were analyzed by IPA software (Kramer et al. 2014). The differentially expressed genes were firstly uploaded into "Dataset Files" of the IPA. By performing the core analysis, only the limiting value of signal transduction pathways and physiological processes with z-score > 2 were obtained from the modules of "Diseases and Bio-functions" through comparison analysis.

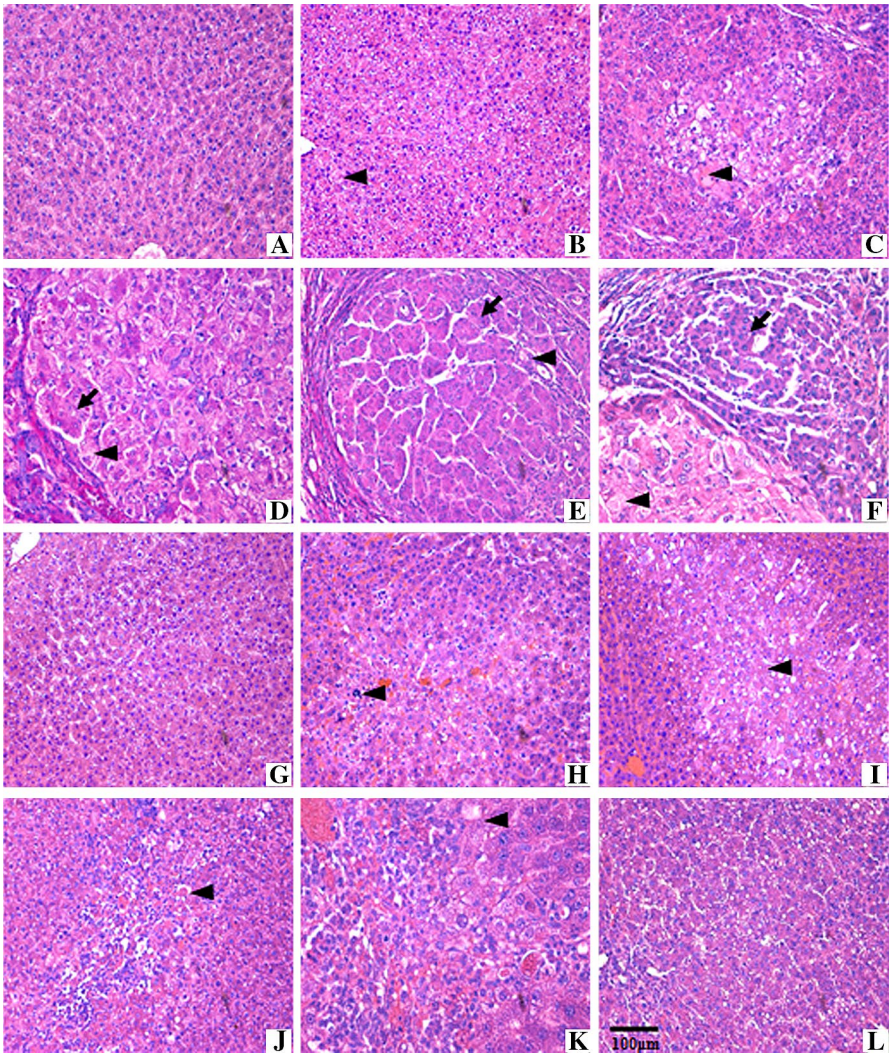

Fig. 1 Histopathological changes of liver tissues obtained from model of rats at 0 (**a**), 5 (**b**), 8 (**c**), 12 (**d**), 16 (**e**), and 18 w (**f**) in HCC as well as 3 (**g**), 6 (**h**), 12 (**i**), 24 (**j**), 48 (**k**), and 72 h (**l**) in AHF (Scale bar =100 μm). The arrows represent "nodule in nodule" phenomena, and the triangles represent hepatocyte necrosis

Results

Histopathological Changes of Liver Tissues in AHF and HCC

In this study, to survey the changes of liver tissues in a larger field, histopathological changes of AHF and HCC were observed with 200-fold magnification, which was different from the 400-fold magnification in our previous study (Wang et al. 2016).

For normal liver of rat, hepatic lobule structure was clear and hepatocytes were closely arranged with clear nucleus and uniformly distributed cytoplasm (Fig. 1a). After treated with DENA for 5 weeks, hydropic degeneration began to be observed in some areas of liver tissue (Fig. 1b). In the 8th week, basophilic or hydropic degeneration of hepatocytes was clearly visible, and hepatocytes began to proliferate (Fig. 1c). At 12, 16, and 18 weeks of HCC, hepatocytes emerged with significant swelling and proliferation, and typical "nodule in nodule" phenomena were obviously observed (Fig. 1d–f). Moreover, hepatocyte necrosis was serious, and cavity and congestion were formed after cell death at the 18th week of HCC (Fig. 1f). At the 3 h of AHF, the slight hydropic degeneration of liver cells was present (Fig. 1g). After treated with CCl_4 for 6, 12, 24, and 48 h, massive necrosis and bridging necrosis appeared in liver tissues, indicating that hepatic lobule was seriously damaged (Fig. 1h–k). At 72 h, the hepatic lobule injury was obviously restored, and the non-necrotic cells arose without 'bubble-like' steatosis (Fig. 1l).

Validation of Chip Results by Real-Time RT-qPCR

To verify the results of the microarray analysis, RT-qPCR assays were used to detect the expression changes of several genes including *Myc, Ccnd1, Ccl2*, and *Spc24* in AHF and *Myc, Ccnd1, Alpl*, and *Ccl2* in HCC. As shown in the following results (Fig. 2), although some genes displayed different expression profiles measured by RT-qPCR and microarray, the expression trends of other genes detected by two methods were basically consistent, demonstrating that the array detection results were relatively reliable. Particularly, the detection results of *Ccl2* displayed different expression profiles measured by above two methods in both AHF and HCC, which showed the complex relationships between the expression of *Ccl2* and the development of diseases (Bianconi et al. 2018). Moreover, the major reason for the considerable discrepancy may be regional specificity of the expression of *Ccl2*. The obtained liver tissues for microarray assay came from the middle part of right lobe, while those for RT-qPCR were from the near middle part of right lobe.

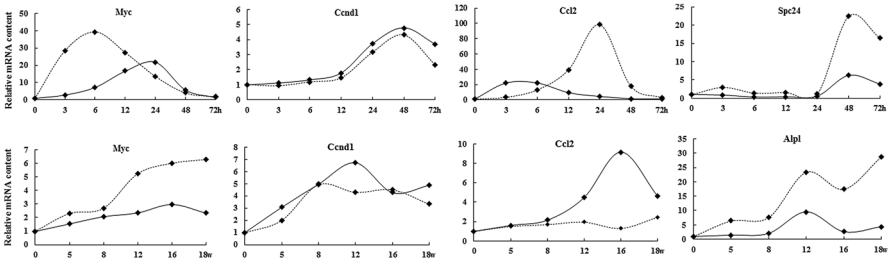

Fig. 2 Verification of gene expression in two kinds of liver diseases by real-time RT-qPCR. The results of RT-qPCR and Rat Genome 230 2.0 Array are presented as a real line and a dotted line, respectively. Time points of AHF are represented by 3, 6, 12, 24, 48, and 72 h; and 5, 8, 12, 16, and 18 weeks denote those of HCC

Table 2 Rat Genome230 2.0 microarray detection data of Reg signaling pathway-related genes in AHF and HCC

Gene symbol	Progression of HCC (hour)						Progression of AHF (week)				
	3 h	6 h	12 h	24 h	48 h	72 h	5 weeks	8 weeks	12 weeks	16 weeks	18 weeks
Akt3	1.4	0.6	1.1	0.9	1.5	**2.3**	**2.1**	0.8	**2.8**	**3.0**	1.8
Bcl2	0.7	0.9	*0.3*	0.6	0.7	0.8	0.9	0.9	1.4	1.5	1.2
Bcl2l1	**2.6**	**4.5**	**2.3**	1.2	1.5	1.2	1.3	1.4	1.7	1.7	1.7
Ccnd1	0.9	1.2	1.5	**3.2**	**4.3**	**2.3**	2.0	**4.9**	**4.3**	**4.5**	**3.3**
Dact1	0.8	*0.3*	*0.5*	*0.4*	0.6	0.7	0.8	0.9	1.2	1.3	0.8
egfr	1.1	1.1	0.9	0.6	0.5	0.7	0.9	0.7	0.5	*0.4*	0.5
Extl3	*0.3*	0.7	0.6	0.7	0.9	1.0	*0.5*	*0.1*	1.2	1.2	1.0
Fos	**75.9**	**21.6**	**32.9**	**9.0**	**4.7**	**2.2**	1.7	**2.4**	**6.2**	**6.5**	**7.4**
Fosl1	2.0	**26.5**	**16.9**	**7.3**	0.9	0.7	0.6	0.8	1.0	0.9	1.0
Fosl2	**20.2**	**22.2**	**12.1**	**4.7**	1.3	1.0	1.7	**3.1**	**11.6**	**9.8**	**10.3**
Hras	1.9	1.3	1.5	1.4	**2.5**	**2.3**	1.7	1.7	1.6	1.7	2.0
Ikbkb	**2.2**	**6.5**	**2.5**	**3.5**	1.7	**3.9**	**2.2**	**3.2**	**3.9**	**2.2**	**2.2**
Il1a	1.0	0.8	0.9	1.1	0.6	0.5	0.7	0.5	*0.4*	*0.3*	*0.3*
Il10	**2.4**	**3.8**	**6.8**	**4.8**	0.5	*0.4*	1.7	0.5	1.5	0.5	0.6
Il1b	*0.5*	1.3	**2.5**	**2.2**	1.1	0.8	0.9	0.8	0.8	1.4	0.8
Il6	1.1	1.3	**3.4**	**2.5**	*0.4*	*0.4*	0.7	0.8	1.1	1.9	0.9
Itpr3	**3.9**	**2.0**	1.0	**2.7**	**2.1**	**4.6**	**2.9**	**4.5**	**7.0**	**8.6**	**6.5**
Jun	**19.7**	**13.0**	**9.9**	**3.9**	1.4	1.1	2.0	**2.3**	**2.5**	**2.6**	**2.1**
Junb	**11.9**	**12.6**	**6.9**	**2.5**	1.6	0.9	1.0	1.5	**2.4**	**2.0**	1.5
Jund	**4.8**	**10.4**	**11.7**	**5.0**	1.5	**2.1**	**2.3**	1.4	**3.3**	**6.4**	**5.7**
Kras	1.1	**2.1**	1.8	1.5	1.5	1.1	1.3	1.2	1.3	1.6	1.3
Mapk3	1.6	1.1	1.7	1.3	**2.9**	1.7	1.9	1.4	**2.5**	**2.8**	**2.7**
Mapk4	**2.4**	**3.3**	**2.8**	2.0	**2.6**	**2.3**	**2.4**	1.4	**2.8**	1.9	1.8

Table 2 (continued)

Gene symbol	Progression of HCC (hour)						Progression of AHF (week)				
	3 h	6 h	12 h	24 h	48 h	72 h	5 weeks	8 weeks	12 weeks	16 weeks	18 weeks
Mapk6	1.4	**2.2**	1.8	1.3	0.7	0.7	0.9	0.9	0.6	0.5	0.6
Mapk7	**2.9**	1.3	1.6	1.3	**2.4**	1.8	2.0	1.2	1.0	1.0	2.0
Mapk9	0.7	*0.5*	0.5	*0.4*	0.5	0.6	0.7	*0.4*	0.6	*0.5*	0.6
Mapk10	**6.6**	**4.4**	0.6	1.1	**2.0**	0.7	**2.1**	1.8	**2.6**	1.6	**4.6**
Mapk11	**2.1**	1.9	1.8	**2.7**	2.0	**2.0**	**2.1**	**2.1**	**2.9**	**2.1**	**2.2**
Mapk13	**2.0**	2.3	**2.1**	**2.3**	1.5	0.9	1.9	1.8	**2.4**	**3.8**	0.8
Mapk14	1.3	2.1	1.3	0.9	1.1	1.1	1.1	1.1	0.9	0.9	0.9
Map2k1	0.8	1.4	1.6	**2.1**	1.2	0.8	0.8	0.9	1.0	1.1	1.0
Map2k3	1.1	**3.6**	1.9	0.9	1.1	1.1	0.7	0.7	0.9	0.8	0.9
Map2k6	1.3	*0.4*	*0.2*	0.5	0.8	**2.1**	1.7	**2.4**	**3.9**	**3.9**	**2.8**
Map3k1	1.9	1.6	0.9	0.9	0.9	1.0	1.5	**2.1**	**2.7**	**2.8**	1.9
Map3k4	0.7	*0.5*	0.6	0.7	0.9	0.8	1.1	1.2	0.9	0.9	0.9
Map3k6	**2.6**	**6.1**	**4.6**	**3.2**	**2.6**	1.3	1.4	1.6	**2.6**	**3.2**	**2.4**
Map3k8	**2.2**	**2.8**	**2.0**	1.3	0.8	0.5	0.8	0.9	0.7	1.1	0.6
Myc	**28.5**	**39.2**	**27.3**	**13.6**	**4.1**	1.8	**2.3**	**2.7**	**5.2**	**6.0**	**6.3**
Mras	*0.4*	0.5	1.0	1.4	1.3	1.1	1.0	0.7	1.6	1.3	1.2
Nfkb1	1.2	**3.5**	**2.1**	1.7	1.1	0.9	1.1	1.3	1.4	2.0	1.3
Nfkb2	**2.5**	**3.8**	**3.2**	**3.2**	1.9	1.4	1.6	**2.2**	**2.3**	**3.1**	**2.2**
Nfkbia	**2.5**	**2.5**	**2.9**	1.3	1.3	0.8	0.9	1.2	1.1	1.5	1.3
Nfkbib	**2.0**	**2.8**	**2.4**	**2.4**	1.3	1.2	1.2	1.2	1.3	1.3	1.2
Nr0b2	0.8	*0.2*	*0.2*	*0.1*	*0.3*	1.1	0.8	1.7	0.9	0.7	0.6
Nr2c2	0.6	0.9	0.6	0.6	0.8	0.7	*0.4*	0.6	1.5	1.5	0.5
Pak1	0.9	1.4	1.3	1.3	1.4	1.2	1.4	1.9	**2.1**	**2.2**	**2.5**

Table 2 (continued)

Gene symbol	Progression of HCC (hour)						Progression of AHF (week)				
	3 h	6 h	12 h	24 h	48 h	72 h	5 weeks	8 weeks	12 weeks	16 weeks	18 weeks
Pak2	0.6	0.7	0.9	1.1	1.1	0.9	0.7	*0.3*	1.3	1.2	1.2
Pak4	1.6	1.8	1.7	1.8	1.8	1.4	1.8	2.0	2.0	**2.1**	1.8
Pik3ca	0.8	**2.6**	1.5	1.0	0.8	0.8	1.0	1.0	1.0	1.1	1.0
Pik3c2g	0.6	*0.4*	*0.3*	*0.2*	*0.3*	*0.4*	0.9	0.7	0.6	0.6	0.5
Pik3r1	1.1	**4.6**	**2.5**	0.7	1.0	0.9	1.2	0.8	1.0	0.9	0.8
Pik3r4	0.7	*0.3*	*0.4*	0.5	0.6	0.7	0.9	0.9	0.8	0.7	0.7
Plcb1	1.2	0.7	*0.4*	0.6	0.6	0.9	0.9	0.9	0.6	0.6	0.6
Plcb4	**2.1**	1.1	1.4	0.9	1.1	0.9	1.1	0.8	**3.4**	**3.9**	**2.1**
Plcd3	**3.6**	**3.0**	1.7	**2.1**	1.4	**3.2**	**3.1**	**3.7**	**2.4**	**3.8**	**2.3**
Plcd4	1.9	**3.0**	1.2	**3.0**	**2.4**	0.7	1.8	**3.6**	**2.5**	**5.4**	**6.4**
Prkch	1.2	1.9	1.6	**2.2**	2.0	1.5	1.3	1.6	2.0	**2.4**	1.9
Prkcz	1.1	0.6	1.9	**2.4**	1.6	1.1	1.1	1.7	1.9	1.7	**2.1**
Prkd3	*0.5*	*0.5*	*0.4*	0.6	0.7	0.7	1.0	0.9	0.8	0.8	0.7
Rac1	**2.4**	**2.7**	**2.6**	**2.5**	1.9	**2.2**	**2.5**	**2.2**	**2.1**	**3.6**	**2.3**
Rras	1.2	1.3	1.2	1.7	1.3	1.2	1.3	1.4	1.9	1.8	**2.2**
Rras2	0.7	**2.1**	1.8	1.8	1.3	1.0	0.9	1.1	1.5	1.2	1.5
Rela	1.8	**2.5**	**2.0**	1.4	1.1	1.0	1.3	1.5	1.4	1.5	1.5
Reg3a	0.7	1.5	0.9	0.7	1.1	**2.1**	**2.3**	**5.8**	1.9	**4.2**	1.3
Reg3b	0.8	1.2	1.2	1.9	0.9	0.9	0.7	0.7	**2.8**	**2.9**	1.5
Reg3g	1.8	**7.7**	**2.6**	**2.4**	**2.9**	**2.4**	1.9	**2.9**	**11.0**	**11.9**	**3.6**
Socs1	1.7	1.7	1.9	1.2	1.7	**2.4**	1.6	1.1	1.2	1.8	1.2
Socs2	0.9	0.9	*0.3*	*0.3*	0.9	0.8	0.6	*0.1*	*0.3*	*0.3*	*0.3*
Socs3	1.6	**4.9**	**2.2**	1.1	1.1	0.6	0.7	0.6	1.3	1.1	0.8

Table 2 (continued)

Gene symbol	Progression of HCC (hour)						Progression of AHF (week)				
	3 h	6 h	12 h	24 h	48 h	72 h	5 weeks	8 weeks	12 weeks	16 weeks	18 weeks
Shc1	1.2	**2.2**	1.9	1.4	1.2	1.4	1.5	1.4	1.4	1.7	1.6
Shc2	0.9	1.4	1.6	1.0	1.3	0.9	1.6	1.7	**2.2**	1.5	1.5
Stat1	*0.5*	*0.5*	*0.4*	1.1	0.8	0.9	1.5	1.1	0.7	1.0	0.8
Stat3	**2.9**	**3.6**	1.8	1.5	1.5	1.3	1.4	1.1	1.5	1.7	1.7
Stat6	**2.2**	1.7	1.2	1.6	2.0	1.7	1.8	**2.1**	**2.7**	**2.6**	**2.3**
Src	2.0	**2.1**	**2.2**	**2.4**	**2.2**	1.5	**2.1**	**2.9**	**3.8**	**5.2**	**2.8**
Taok2	1.3	1.3	1.1	1.0	1.2	1.0	1.7	1.7	**2.1**	**2.3**	1.7
Tcf4	1.2	1.2	1.0	1.4	1.4	1.3	1.6	1.9	**2.4**	**3.2**	**2.2**
Tp53	1.8	**2.9**	**2.4**	**2.7**	**2.1**	1.9	**2.3**	**2.0**	**2.2**	**2.4**	**2.6**
Tnf	0.8	1.7	1.9	**2.2**	0.8	0.7	0.9	0.9	1.1	1.3	1.0

The values ≥ 2 are marked with bold ground and represent significantly up-regulated expression of genes; those ≤ 0.5 are marked with italic ground and represent significantly down-regulated expression of genes

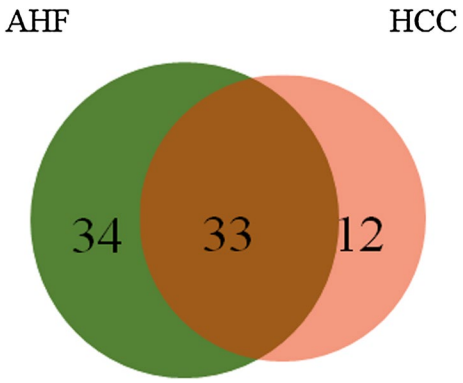

Fig. 3 Venn diagram showed the significantly expressed genes related to the Reg signaling pathways during the occurrence of AHF and HCC

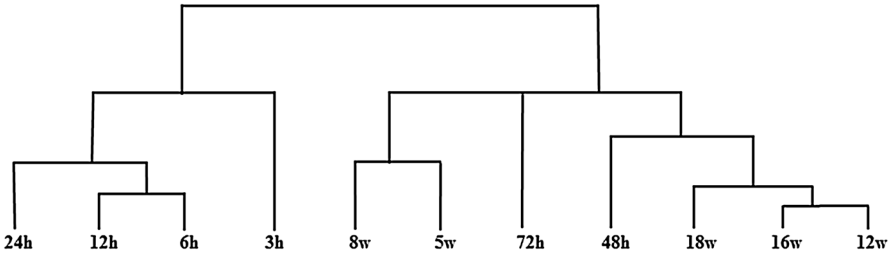

Fig. 4 A cladogram was produced by complete array expression values of the Reg signaling pathways-related genes in AHF and HCC

Comparison of Expression Profiles of the Reg Signaling Pathways-Related Genes in AHF and HCC

In this study, Rat Genome 230 2.0 Array was used to detect the genome-wide gene expression profiles during the occurrence of AHF and HCC, and the obtained microarray data have been submitted to the Gene Expression Omnibus database with the accession numbers of GSE73494 and GSE73498, respectively.

KEGG, QIAGEN, and literature were mainly applied to obtain 140 genes of the Reg-related signaling pathways, which were selected for further analysis for AHF and HCC. As a result, a total of 79 genes were found to be significantly changed in two kinds of liver diseases (Table 2). And among them, 67 genes including 54 up-regulated and 13 down-regulated, and 45 genes with 38 up-regulated and 7 down-regulated were related to the occurrence of AHF and HCC, respectively (Fig. 3). These results suggested that the up-regulated genes were more than the down-regulated ones in AHF and HCC.

To compare the Reg signaling pathways-related genes expression profiles between AHF and HCC, H-clustering analysis was carried out. Based on the expression similarity presented by the dendrogram, the results found that 48 and 72 h of AHF and 5, 8, 12, 16, and 18 weeks of HCC were clustered together, whereas 3, 6, 12, and 24 h of AHF were clustered together (Fig. 4). It was clear that some time points of AHF were always highly correlated with those of HCC, though their gene expression profiles were different on the whole, suggesting that there were some similarities between the recovery of AHF and the occurring of HCC regulated by Reg family.

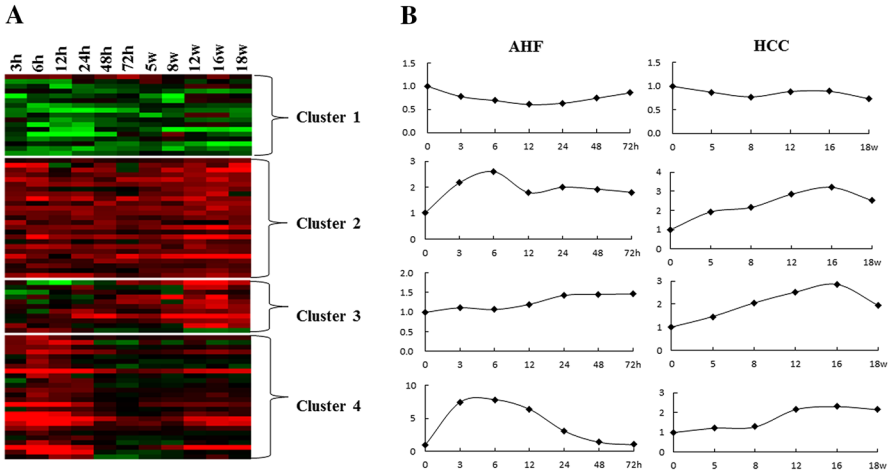

Fig. 5 Global comparison of expression patterns of Reg signaling pathways-related genes between AHF and HCC. (**a**) K-means clustering of 79 genes. Red and green colors show the higher and lower expression levels than the control, respectively. (**b**) Differences in 4 clusters between AHF and HCC (Color figure online)

For further elucidating the similarities and differences between transcriptional patterns of AHF and HCC, we looked up the expression changes of 67 AHF-related genes in HCC and 45 HCC-related genes in AHF. Then, a total of 79 genes were obtained with expression values at 11 time points (3, 6, 12, 24, 48, and 72 h after CCl_4 treatment and 5, 8, 12, 16, and 18 weeks after DENA treatment). Subsequently, 79 genes above were categorized into 4 clusters by K-means. As shown in Fig. 5, cluster 1 contained 17 genes that were significantly down-regulated in both AHF and HCC. Cluster 2 included 25 genes, which displayed increased expression with a peak at 6 h during the occurrence of AHF and peaked at 16 w in HCC. There were 11 genes classified into cluster 3 with slightly down-regulated expression in AHF and up-regulated in HCC. Twenty six genes were embodied in cluster 4, which was gradually up-regulated and reached its expression peak at 6 h of AHF, but exhibited non-significantly up-regulation during HCC.

Functional Enrichment Analysis of the Reg Signaling Pathways-Related Genes in AHF and HCC

According to functional annotation categories in DAVID analysis, the Reg signaling pathways-related genes in AHF and HCC were involved in various physiological activities including stress response, signaling transduction, cellular response, inflammatory response, aging, material metabolism, cell cycle, apoptosis, etc. To determine whether some GO categories are over-represented or not, the over-represented GO categories with EASE scores of < 0.05 were selected out (Table 3). Obviously, cluster 1 (C1) was enriched with the genes in categories of stress response, and signal transduction-related genes were predominant in cluster 2 (C2) and cluster 3 (C3). Genes related to stress response, inflammatory response, cell cycle, cell proliferation, and cell apoptotic were involved in cluster 4 (C4).

Table 3 Over-represented functional categories in 4 clusters of Reg signaling pathways-related genes in AHF and HCC

Enriched biological process	*P* value	Enriched biological process	*P* value
Cluster 1		Response to mechanical stimulus	6.70E−11
Response to peptide hormone	3.60E−06	Response to cAMP	7.40E−10
Response to organic cyclic compound	1.50E−03	Response to glucocorticoid	2.00E−08
Response to cytokine	3.20E−03	Inflammatory response	2.30E−06
Positive regulation of gene expression	3.50E−03	Acute inflammatory response	4.50E−04
Cluster 2		Cellular response to interleukin-1	8.90E−06
MAPK cascade	9.30E−12	Cellular response to cytokine stimulus	2.90E−05
Protein phosphorylation	8.40E−07	Cellular response to hepatocyte growth factorstimulus	2.40E−04
Intracellular signal transduction	3.00E−06	Cellular response to tumor necrosis factor	7.70E−04
Ras protein signal transduction	3.80E−05	Cellular response to lipopolysaccharide	3.70E−11
Peptidyl-serine phosphorylation	4.40E−04	Regulation of cell cycle	5.80E−04
Cluster 3		Positive regulation of transcription, DNA-templated	2.40E−12
Protein phosphorylation	4.20E−06	Lipopolysaccharide-mediated signaling pathway	9.90E−06
Intracellular signal transduction	1.10E−03	Intrinsic apoptotic signaling pathway in response to DNA damage	5.00E−05
MAPK cascade	1.30E−03	Cytokine-mediated signaling pathway	8.60E−04
Cellular response to insulin stimulus	2.10E−03	MAPK cascade	4.10E−11
Cluster 4		Activation of MAPK activity	1.60E−04
Response to cytokine	4.90E−15	Protein phosphorylation	5.10E−04
Response to organic cyclic compound	2.10E−14	Transcription from RNA polymerase II promoter	7.30E−07
Response to drug	2.70E−14	Glucose metabolic process	1.80E−06

P value represents EASE score (a modified Fisher's exact test)

Predicted Regulatory Effects of the Reg Signaling Pathways-Related Branches in AHF and HCC

To clarify which branch may play more important roles during AHF and HCC, pathway analyses were performed to connect the differentially expressed genes with canonical biological pathways by IPA software. Therefore, we analyzed 79 genes that were differentially expressed during rat AHF and HCC by using IPA software, and seven significantly enriched branches were shown in Fig. 6. Obviously, seven branches including the JAK/STAT, NF-κB, ERK1/2, P38, JNK, PLC, and PI3K/AKT signaling pathways were all activated during AHF and HCC. Moreover, the

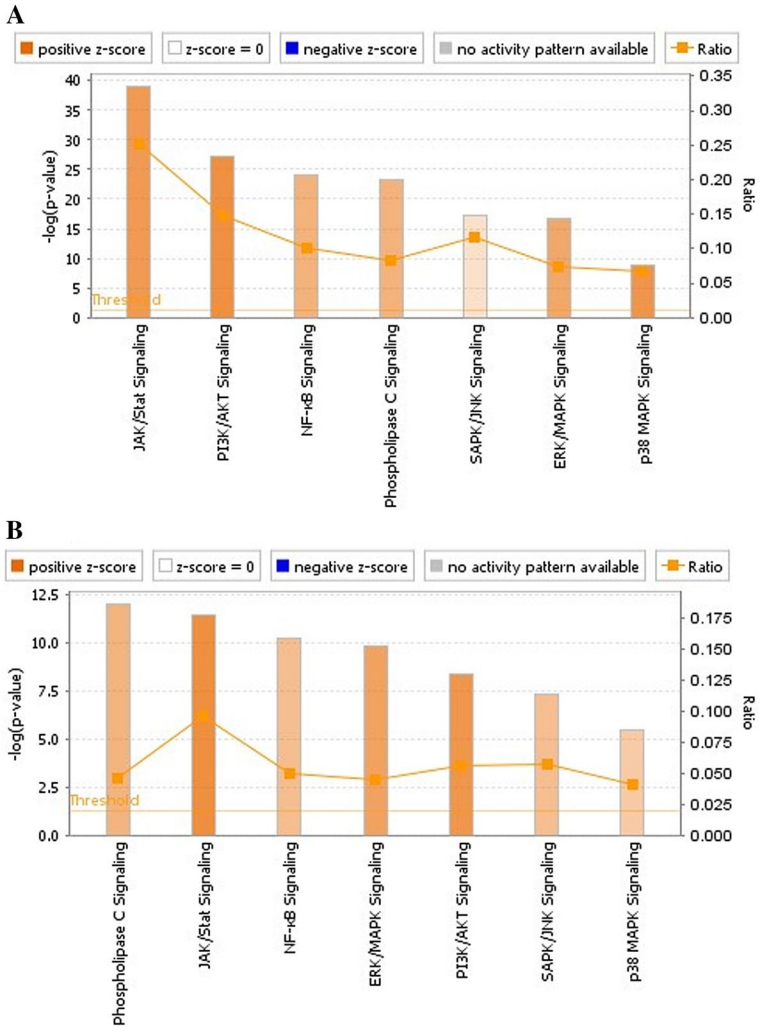

Fig. 6 IPA analysis showed that the Reg-mediated canonical pathways were significantly enriched during rat AHF (**a**) and HCC (**b**). Each histogram represents a particular pathway/branch

JAK/STAT and PI3K/AKT signaling pathways had more effects on AHF, while the PLC signaling pathway had more affected on HCC. Three signaling pathways related to MAPK ranked at last, indicating that they may play less important role in the development of AHF and HCC.

To further clarify which physiological functions may play important roles during the occurrence of AHF and HCC, a total of 79 genes were uploaded to IPA software for core analysis and comparison analysis, and bio-functions related with the Reg signaling pathway in AHF and HCC were obtained (Fig. 7). Obviously, seven branches including the Reg-mediated JAK/STAT, NF-κB, ERK1/2, P38, JNK, PLC, and PI3K/AKT signaling pathways mainly participated in cell proliferation, cell cycle, inflammation, apoptosis, synthesis of DNA, cell death, and so on. Majority of the signaling pathways, except the Reg-mediated PLC signaling pathway, participated in the process of apoptosis in AHF and HCC. The Reg-mediated JAK/STAT signaling pathway mainly activated apoptosis, but apoptosis was inhibited by the NF-κB, ERK1/2, JNK, and PI3K/AKT signaling pathways in AHF and HCC. The Reg-mediated JAK/STAT, NF-κB, ERK1/2, and JNK signaling pathways markedly inhibited cell death in HCC. The Reg-mediated JAK/STAT, JNK, and PLC signaling pathways may regulate cell cycle in both AHF and HCC. Clearly, the PLC signaling

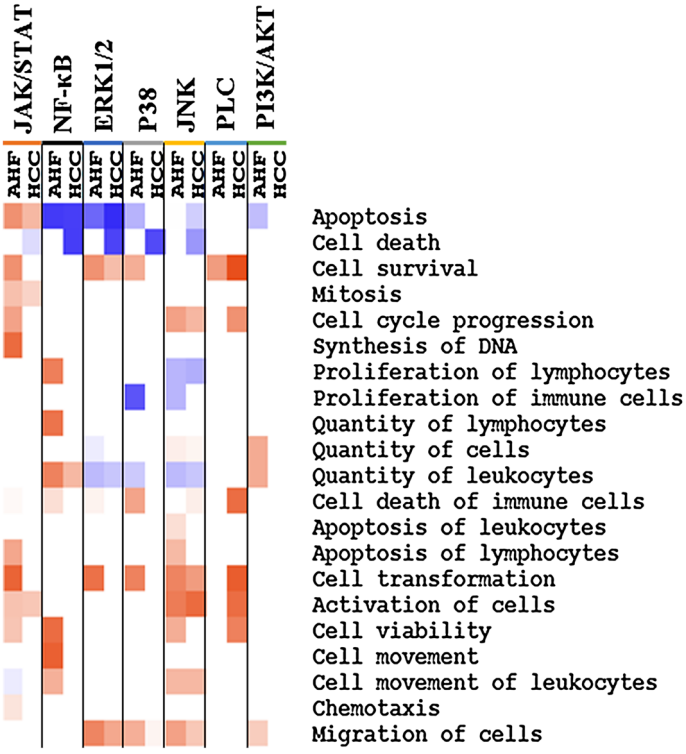

Fig. 7 Biofunction heatmap analysis of the Reg-mediated JAK/STAT, NF-κB, ERK1/2, P38, JNK, PLC, and PI3K/AKT signaling pathways-related genes in AHF and HCC by IPA software

pathway modulated cell proliferation and inflammation in HCC. The Reg-mediated NF-κB and PI3K/AKT signaling pathways were closely related to inflammation in AHF, while the JNK signaling pathway was involved in regulating cell proliferation in HCC. NF-κB, PI3K/AKT and three pathways of MAPK including ERK1/2, P38, and JNK may participate in regulating the proliferation of various inflammatory cells.

Discussion

To uncover the close relationships between the expression profiles of Reg-relate genes and the occurrence of AHF and HCC, this study used Rat Genome 230 2.0 Array to detect the expression profiles of 140 genes related to the Reg signaling pathway in AHF and HCC. It was found that 67 and 45 genes were significantly changed during the occurrence of AHF and HCC, respectively. DAVID functional analysis indicated that the Reg-related genes were involved in stress response, inflammatory response, signaling transduction, and regulation of cell cycle. Treeview and DAVID analyses pointed out that stress response was slightly more strengthened in AHF than that in HCC. Wu et al. found that endoplasmic reticulum (ER) stress response was observed in nearly all physiologies related to acute and chronic liver diseases (Wu et al. 2016). Therefore, it needs to be further studied whether other types of stress responses modulate the occurrence and development of AHF and HCC.

In this study, IPA software was further utilized to predict activities of enriched bio-processes and potential signaling pathways during AHF and HCC, and we found that the Reg-mediated JAK/STAT, NF-κB, ERK1/2, P38, JNK, and PI3K/AKT signaling pathways were actually crucial to AHF, and the Reg-mediated PLC signaling pathway was more closely related to HCC. Apoptosis and cell death have been recognized as two critical physiological phenomena in AHF and HCC (Yoshikawa et al. 2001; Riordan and Williams 2003). This study found that apoptosis modulated by Reg family was basically weakened, but the Reg-mediated JAK/STAT signaling pathway promoted apoptosis in both AHF and HCC. In accordance, Reg Iα protein was found to induce β cell apoptosis through STAT signal in pancreas (Nakagawa et al. 2013). Moreover, the Reg-mediated NF-κB and MAPK pathways were involved in inhibiting apoptosis and cell death in HCC. Reg Iα was found to induce the MKP-1 activation by regulating MAPK phosphorylation and ultimately led to decreased apoptosis and death of pancreatic-derived cells (Wu and Bennett 2005; Mueller et al. 2008). A previous study showed that Reg Iα mediated anti-apoptosis by NF-κB and MAPK in pancreatic acinar AR4–2J cells (Malka et al. 2000), which indicated that Reg family could mediate anti-apoptosis through the MAPK pathways. In this study, Reg signaling pathways-related genes including Reg3a, Reg3b, and Reg3g were found to be up-regulated in AHF and HCC. Thus, most of the Reg family-mediated pathways except JAK/STAT pathway account for the inhibition of apoptosis and cell death in HCC.

Cell proliferation mainly participates in the occurrence of HCC and AHF (Russo and Parola 2011; Hou et al. 2017). This study found that the Reg-mediated JNK pathway regulated cell cycle, mitosis, and DNA synthesis in both AHF and HCC.

Consistently, Reg Iα protein bound to MKP-1, regulated cyclin D, and was involved in cell cycle by activating the JNK signal transduction pathway in pancreatic-derived cells (Mueller et al. 2008). This study found that Reg3a (REG3A) was up-regulated in rat AHF and HCC, and the Reg-mediated PLC and JAK/STAT pathways had more effects on HCC in cell cycle progression. Accordingly, Liu et al. demonstrated that overexpression of REG3A acted as a key molecular for the upregulation of the JAK2/STAT3 pathway, and accelerated cell cycle progression by promoting cyclin D1 expression in inflammation-related pancreatic cancer development (Liu et al. 2015). Therefore, JNK and JAK/STAT pathways may be involved in modulating cell cycle progression in HCC.

Inflammation plays a vital role in the pathogenesis of AHF and HCC (Donnelly et al. 2016; Ringelhan et al. 2018). In this study, the result of DAVID showed that the genes associated with inflammatory response were enriched in cluster 4, and we speculated that inflammatory response was enhanced in the early phase of AHF. Accordingly, IPA analysis predicted that the quantity and proliferation of lymphocytes and leukocytes cells mediated by NF-κB pathway were increased in the occurrence of AHF (Fig. 7), which may be associated with the intense stimulation of CCl_4. Furthermore, Gironella et al. revealed that Reg mediated its anti-inflammatory effects by inhibiting the NF-κB pathway in inflammatory bowel disease (Gironella et al. 2005). Thus, it is indicated that the Reg-mediated NF-κB signaling pathway may act as a critical pathway in regulating inflammation during AHF. Inflammation was increased by the Reg-mediated JNK and P38 signaling pathways, but cell quantity and proliferation of lymphocytes and leukocytes cells regulated by the above two pathways were decreased, which deserves further study.

REG3A enhanced the migratory and invasive of cancer cells through activation of the JAK/STAT pathway (Nigri et al. 2017). However, this study found that Reg3a was up-regulated in AHF and HCC, but the obvious effects of the Reg-mediated JAK/STAT pathway on cell migration were not observed by IPA analysis, which was not consistent with the above-mentioned result from Nigri et al. The role of the Reg-mediated JAK/STAT pathway should be further studied in AHF and HCC. Increasing studies have revealed that the NF-κB and P38 pathways were involved in inflammation-mediated hepatocarcinogenesis (Hsieh et al. 2007; Nakagawa and Maeda 2012). Coulombe et al. have reported that inflammation is induced by regulating the NF-κB and PI3K/AKT pathways in HCC (Coulombe and Rivard 2016). This study demonstrated that the Reg-mediated NF-κB, P38, and PI3K/AKT pathways took part in inflammation during the occurrence of AHF, but had no effects on HCC, implying that Reg family may not be involved in activating the above pathways during inflammation in HCC. However, our study showed that cell migration and leukocytes movement regulated by JNK were enhanced in HCC. Therefore, the Reg-mediated JNK pathway may play a crucial role in inflammation during HCC.

In conclusion, a total of 140 genes were identified to be related to Reg family, and 79 genes from them were differentially associated with AHF and HCC occurrence. According to GO annotation and IPA analysis,the Reg-mediated MAPK (ERK1/2, JNK, P38) and NF-κB signaling pathways were mainly involved in inhibiting cell death in HCC. Additionally, the Reg-mediated JAK/STAT and JNK pathways may regulate cell cycle in HCC. Our data also suggested that the Reg-mediated NF-κB,

P38, and PI3K/AKT pathways were involved in inflammation during the occurrence of AHF, but had no effect on HCC. However, these conclusions were drawn mainly based on the microarray data. In order to confirm that these physiological processes are truly dependent upon Reg family, experiments of Reg overexpression or knockdown are needed to perform for further investigating the regulatory effects of Reg signaling pathways in AHF and HCC.

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Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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