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ORIGINAL ARTICLE

Comparative Analysis of Regulatory Role of Notch Signaling Pathway in 8 Types Liver Cell During Liver Regeneration

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Abstract

Notch signaling is closely related to cell proliferation, cell apoptosis, cell fate decisions, DNA damage repair, and so on. However, the exactly regulatory mechanism of Notch signaling pathway in liver regeneration (LR) remains unclear. To reveal the role of Notch signaling pathway in rat liver regeneration, Ingenuity Pathway Analysis (IPA) software and related pathway database were firstly used to construct the Notch signaling pathway in this study. Next, eight type cells with high purity were obtained by Percoll density centrifugation and immunomagnetic beads sorting. Then, the expression profiles of Notch signaling pathway-related genes in eight type cells were checked by using Rat Genome 230 2.0 Array, and the results showed that the expression of 42 genes were significantly regulated. H-cluster results showed that the hepatic stellate cells are attributed to one cluster; hepatocyte cell, oval cell, sinusoidal endothelial cell, and Kupffer cell are clustered together; and biliary epithelial cell, pit cell, and dendritic cell are one cluster. IPA software and Expression analysis systematic explorer analysis indicated that Notch signaling pathway-related genes were involved in cell proliferation, apoptosis, cell cycle, DNA damage repair, etc. In conclusion, Notch signaling pathway might regulate various physiological activities of LR through multiple pathways.

Keywords Liver regeneration · Notch signaling pathway · Ingenuity Pathway Analysis software · Regulatory mechanism

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Introduction

Liver is one of the naturally regenerative organs in the body and performs many important functions such as synthesis and storage of glucose, detoxification, and immune defense (Wu and Tao 2012). After liver injury or partial hepatectomy (PH), a sequence of highly orchestrated cellular events happen to recover liver mass and function (Geisler and Strazzabosco 2015), which is carried out by proliferation of mature hepatocytes, biliary epithelial cells, Kupffer cells, and stellate cells. Increasing data suggest that many factors and multiple signaling pathways are involved in LR.

Notch is an evolutionarily conserved receptor protein, which locates in a variety of cell surfaces to mediate cell signaling transduction after interacting with some ligands. In mammals, four transmembrane Notch receptors (Notch-1, -2, -3, and -4) and two types of ligands, Jagged (Jag-1, -2) and Delta-like proteins (Delta-1, -3, and -4), have been identified. ligand-receptor binding leads to the activation of Notch signaling cascade by γ-secretase complex and release of Notch intracellular domain (NICD), which could be translocated to nuclei and mediate the transcription of Notch target genes, such as Hes and Hey14 (Zhang et al. 2018b).

Notch signaling pathway has pleiotropic effects and could regulate a wide range of events in organism development, including cell proliferation, differentiation, apoptosis, cell fate decisions, and DNA damage repair (Guo et al. 2014). In the past few years, the Notch signaling has been gradually considered as a major player in liver biology and pathophysiology. Immunohistochemical staining of normal rat liver showed that Notch was expressed in hepatocytes, bile duct cells, endothelial cells, sinusoidal cells, and the ligand of Jagged1 was expressed in hepatocytes, bile duct cells (Nijjar et al. 2001). Kohler (Kohler et al. 2004) investigated the expression of Notch and Jagged-1 in rat liver following 2/3 partial hepatocytes, and found that the Notch/Jagged signaling pathway was activated and could potentially promote the liver cells proliferation; furthermore, addition of recombinant Jagged-1 protein to primary cultures of hepatocytes could stimulate hepatocyte DNA synthesis. The proliferation and functional differentiation of regenerating liver in mice was impaired when the common downstream transcription factor of all Notch receptors and transcription factor recombination signal-binding Protein-Jkappa (RBP-J) were conditionally deleted, indicating that Notch signaling might support liver cells proliferation (Wang et al. 2009). During liver development, Notch signaling was activated in hepatoblasts and controlled their differentiation into biliary cells (specific hepatic cell type) (Jeliazkova et al. 2013). We found that the Notch-RBPJ signaling axis critically controlled intrahepatic bile duct cells regeneration by coordinating the fate decision of hepatic progenitor cells, which appeared to play an important role in liver regeneration (Lu et al. 2016). Literature studies showed that the expression of Notch-1/Jagged-1 was markedly induced from 1 day to 5 days after PHx, combining with the increase of the Proliferating Cell Nuclear Antigen (PCNA) and Ki-67 (Zhang et al. 2018a). Villanueva (Villanueva et al. 2012) unveiled that excessive

activation of Notch signaling might be involved in liver malignancies, such as hepatocellular carcinoma and intrahepatic cholangiocarcinoma. In human, mutations in the ligand Jagged1 or its receptor Notch-2 resulted in the paucity of bile ducts and severe cholestasis (Wang et al. 2017). In summary, Notch signaling pathway may play a dual role both in liver regeneration and liver cancer. Therefore, the precise mechanism of Notch signaling pathway in LR needs to be further elucidated.

Liver is composed of at least eight hepatic cell types, including hepatic parenchyma cells (hepatocytes, HCs, and biliary epithelial cells, BECs) and nonparenchymal cells such as HSCs, Kupffer cells (KCs), and sinusoidal endothelial cells (SECs). However, Notch signaling pathway plays a different role in specific hepatic cell types, to understand the regulatory mechanism of Notch in LR, Ingenuity Pathway Analysis (IPA) software and some data websites were employed to construct the networks of Notch signaling pathway. Then, Rat Genome 230 2.0 Array was employed to check the expression changes of Notch signaling pathway-related genes in eight kinds of liver cells during LR. Finally, the expression extremum of abovementioned genes was uploaded to the IPA software to analyze the physiological activities associated with Notch pathways. As far as we know, this is the first time to apply systems biology and pathway analysis software IPA to explore the molecular mechanism of Notch in LR (Yang et al. 2016).

Materials and Methods

Preparation of Rat Regenerating Livers

Adult healthy male SD rats, each weighing 200 ± 10 g, supplied by the Experimental Animal Center of Henan Normal University, were housed in a controlled temperature room (22 ± 1 °C) with a 12:12-h light–dark cycle. A total of 76 adult male rats were randomly divided into 19 groups of 4 rats each, including 9 partial hepatectomy (PH) groups, 9 sham-operated (SO) groups and one control (NC) group. PH groups were subjected to 2/3 PH following the method of Higgins (Sade et al. 2004). All the same, SO group received the same procedure but without liver removal. Four rats were sacrificed at 0, 2, 6, 12, 24, 30, 36, 72, 120, and 168 h after PH, respectively, and their liver tissues from the middle part of the right lobe were instantly removed and stored at − 80 °C for later use. The whole handling procedures were strictly compliant with the current Animal Protection Law of China and the studies have been approved by a research ethics committee at the university.

Isolation and Purification of Eight Hepatic Cell Types

The isolation of eight hepatic cell types at 10 time points after PH was performed as described in our previous study (Wang and Xu 2010). Collecting the mixed cell populations, which have a separation efficiency $\geq 5.0 \times 10^8$ cells/rat, cell viability $\geq 95\%$, and red cells $\leq 0.1\%$. 6 mL of the acceptable mixed cell populations was

obtained through 60% Percoll density gradient centrifugation, and the harvested pellet at bottom and supernatant was the purified hepatocytes (HCs) and nonparenchymal cell-enriched supernatant fractions, respectively (Duret et al. 2007; Vondran et al. 2008). Then, the mixed nonparenchymal cells were incubated with 10 mL/mL of rat anti-CK19-polyethylene (PE), anti-THY1-PE, anti-GFAP-PE, anti-CK31-PE, anti-CD68-PE, anti-CD161a-PE, or anti- CD11c-PE (all antibodies from Miltenyi Biotec) for 15 min at 4 °C, and then with 10 mL/mL of rat anti-PE MACS microbeads (Miltenyi Biotec) for another 15 min. Finally, cell suspension was loaded onto the separation column and allowed to flow naturally. After the magnetic field was removed, the obtained solutions washed by PBS buffer at 4 °C were the suspension for BECs, OCs, HSCs, SECs, KCs, PCs, and DCs, respectively.

Construction of Notch-Mediated Signaling Pathway Network

Notch signaling regulates cell proliferation, apoptosis, DNA damage repair by binding of Notch family receptors (Notch-1, -2, -3, -4) to their cognate ligands such as Jag-1, -2, or Delta-like (Dll-1, -3, -4). Upon ligand binding, Notch receptor was activated and cleaved by the action of γ-secretase, leading to the cytosolic release of the NICD, which migrated into the nucleus where it binds to the transcription factor RBP-Jκ to activate the expression of Notch target genes (Morell et al. 2013). Firstly, the "Notch signaling pathway" was entered into the "Pathway and Function" module of IPA software to obtain the proteins in every corresponding signaling pathway. Then the above results were filtered and integrated by using signaling maps at the databases of GenMAPP, KEGG, BIOCARTA, QIAGEN, and Biocompare, and reconfirmed by pertinent articles.

Rat Genome 230 2.0 Microarray Detection and Data Analysis

Total RNAs were extracted from 0.5 g liver following the manual of TRIzol reagent (Invitrogen Corporation, Carlsbad, California, USA)(Takano et al. 2010). Then, RNA of liver cells was purified according to the protocol as previously described. Subsequently, the purified RNAs were reverse transcribed into cDNA using a SuperScript II Reverse Transcription Kit (Invitrogen, USA), and the second strand RNA was synthesized according to the guidelines for the Affymetrix cDNA kit, which then was followed by cRNA hybridization, microarray wash, image scanning, and data analysis. Finally, the signal intensity was normalized by using Affymetrix GCOS 2.0. The data of each array were initially normalized by scaling all signals to a target intensity of 200. The ratio at each time point was calculated through the normalized signal values of experimental group compared to control group (0 h). When the relative value was $\leq$ 0.5-fold control group, the genes were considered as significantly down-regulated; genes with relative value between 0.5 and 2 were considered as no biologically meaningful expression; genes with relative value $\geq$ twofold were considered as significantly up-regulated. To minimize the experimental operation and analytical error of microarray, each sample was repeated three times, and the average value was used for statistical analysis (Wang et al. 2016a).

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

To verify the chip data, 8 target genes were selected for validation using qRT-PCR. Primer sequences were designed by Primer Express 2.0 software according to mRNA sequences of the 8 genes: *G6pc, Ggt1, Oc2, Gfap, Cd14, Lyz, Cd56, Cd86* (marker genes of HCs, BECs, OCs, HSCs, SECs, KCs, PCs, and DCs, respectively) and the housekeeping gene *β-actin*, and they were all synthesized by Shanghai Generay Biotech Ltd (Shanghai, China). Next, target genes were subjected to quantitative polymerase chain reaction (PCR) amplification using a PRISM 7900 Sequence Detector (Applied Biosystems; Foster City, CA, USA) according to the operational guideline manual for QuantiTect SYBR Green RT-PCR Kit (Qiagen). The relative expression changes of target genes were computed through that of internal control β-actin.

Functional Enrichment Analysis of Notch Signaling Pathway-Related Genes

In order to characterize the expression profiles of the above genes, this study employed hierarchical-means (H-means) to classify the significantly changed genes according to gene expression similarity in different hepatic cell types. And then, the expression extremum of Notch signaling pathway-related genes was loaded into IPA software for core analysis, and the biological function changes in 8 liver cell types of LR were got in "Diseases and Functions" module and analyzed by comparison analysis. And the biological function with z-score > 2 was picked and z-score was used for clustering analysis by H-means (Kramer et al. 2014).

Function Prediction of Every Path of Notch Signaling Pathway in 8 Hepatic Cell Types

To elucidate the regulatory role of Notch signaling pathway in LR, the genes associated with each branch of Notch signaling pathway were respectively summarized. Then, IPA software was utilized to analyze the heatmap of biofunction based on the expression changes of each branch-related genes using comparison analysis. Briefly, the differentially expressed each branch-related gene symbols and their corresponding expression extrema in eight liver cell types were uploaded to the "Dataset Files" of IPA. Then, core analysis and comparison analyses among eight liver cell types were proceeded successively. Next, heatmaps of biofunctions were obtained in the model of "Diseases and Functions," and every heat map square could be clicked to display the network and the possible crucial genes in the network. Finally, only the biofunctions with z-score > 2 were selected to produce ultimate heatmap of biofunctions in every branch.

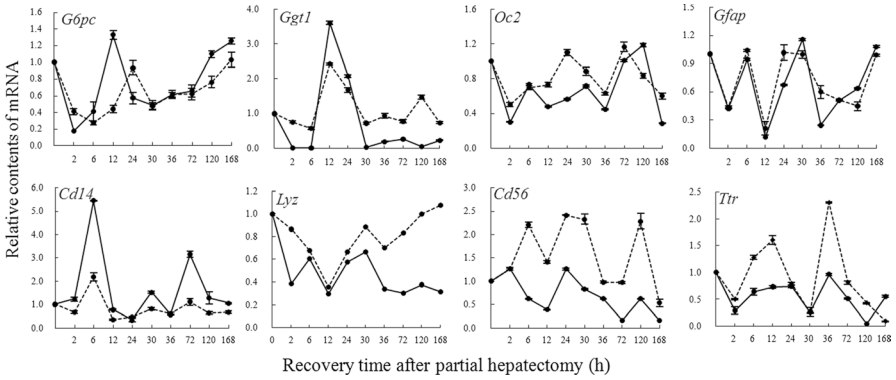

Fig. 1 Verification of gene expression by qRT-PCR in eight hepatic cell types during liver regeneration. The results of qRT-PCR and Rat Genome 2302.0 array are presented as a real line and a dotted line, respectively. *X*-axis represents 10 recovery time points after partial hepatectomy, and *Y*-axis represents relative mRNA abundance of gene

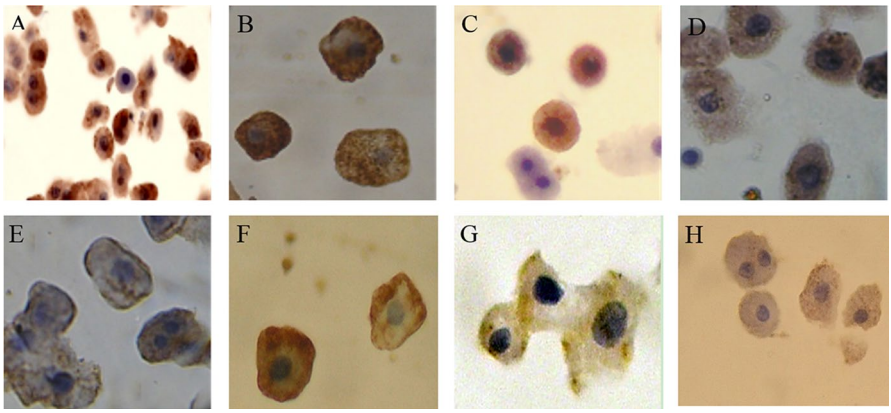

Fig. 2 Immunostaining of eight hepatocyte marker genes during liver regeneration. **a** Immunostaining of ALB in HCs, **b** γ-GT in BECs, **c** OC2 in OCs, **d** Desmin in HSCs, **e** CD56 in SECs, **f** Lysozyme in KCs, **g** CD8 in PCs, **h** CD86 in DCs

Results

Validation of Chip Results by Real-Time RT-PCR

The expression changes of marker genes *G6pc* of HC, *Ggt1* of BEC, *Oc2* of OC, *Gfap* of HSC, *Cd14* of SEC, *Lyz* of KC, *Cd56* of PC, and *Cd86* of DC were detected by RT-PCR(Chang and Xu 2010). Results showed that there was no apparent difference between RT-PCR and Rat Genome 230 2.0 for selected genes, indicating that the results of chip were reliable (Fig. 1). Besides, the extraction quality of the different cell type--specific markers was validated by immunostaining (Fig. 2).

Notch-Mediated Signaling Pathway Network

The interaction network of Notch signaling pathway was summarized by using database such as GenMAPP, KEGG, BIOCARTA, QIAGEN, and Biocompare. Then, the above results were integrated in one diagram. And the networks were double confirmed by some pertinent articles. The results showed that 9 branches were involved in Notch-mediated signaling pathway (Fig. 3). Path 1: Notch—LCK → PI3K → AKT1 → MAP3K5 → MAP2K 3/4 → JNK → BCL2/BCL2L1┤ BAX → CYCS → CASP9 → CASP3 → apoptosis; path 2: Notch—LCK → PI3K → AKT1┤ BAD┤ BCL2L1/BCL2 ┤ BAX → CYCS → CASP9 → CASP3 → apoptosis; path 3: Notch— LCK → PI3 K → AKT1 → NF-κB → BCL2/BCL2L1┤ BAX → CYCS → CASP9 → CASP3 → apoptosis; path 4: Notch—LCK → PI3K → AKT1 → MDM2┤ TP53 → CDKN2A ┤ CDK4/6┤ RB1┤ E2F1 → cellproliferation; path 5: Notch—LCK → PI3K → AKT1 → MDM2┤ TP53 → CDKN1A/B┤ CDK2/4┤ RB1┤ E2F1 → cellproliferation; path 6: Notch—LCK → PI3K → AKT1 → MDM2┤ TP53 → CTNNB1 → J un —Fos → cell proliferation; path 7: Notch —LCK → PI3K → AKT1 → MDM2 ┤ TP53 → CTNNB1 → Myc/Max/Mad → DNA repair; path 8: Notch —LCK → PI3K → AKT1 ┤ GSK3B ┤ CTNNB1 → Jun—Fos → cell proliferation; path 9: Notch—LCK → PI3K → AKT1 ┤ GSK3B┤ CTNNB1 → Myc/Max/ Mad → DNA repair. The expression changes of every path-associated gene were listed in Supplementary Table 1. Above all, Notch might regulate several biological processes including cell proliferation, apoptosis, and DNA damage repair through the above-mentioned paths.

Expression Profiling of Notch Signaling Pathway in 8 Liver Cell Types During LR

Based on the interaction network of Notch signaling pathway, we found that nine branches including 33 proteins and 47 genes were involved in pathways (Wang et al. 2015). Expression profiles of the 47 genes associated with Notch signaling pathways were detected by Rat Genome 230 2.0, and a total of 42 genes were significantly changed (Xu et al. 2013); among them, 25 genes were significantly changed in HC, 38 in BEC, 15 in HOC, 25 in HSC, 24 in SEC, 21 in KC, 27 in PC and 24 in DC. The gene expression peak of HSC was at 2 and 12 h, HOC at 6 h, BEC at 12 h, KC and DC at 24 h, SEC at 6 h and 30 h, HC and PC at 72 h (Table 1). Subsequently, hierarchical clustering (H-clustering) method was employed to comprehensively characterize the expression patterns of the genes related to Notch-mediated signaling pathway during LR according to gene expression similarity (Wang et al. 2015). The number of up-regulated genes in HCs, BECs, HOCs, HSCs, SECs, KCs, PCs, and DCs were more than that of down-regulated, and the expression changes of them were also unanimous. Based on the analysis of gene expression profiles, HSCs were in a single branch; HC, HOC, SEC, and KC were grouped in one cluster; while BEC and PC, DC in another cluster (Fig. 4).

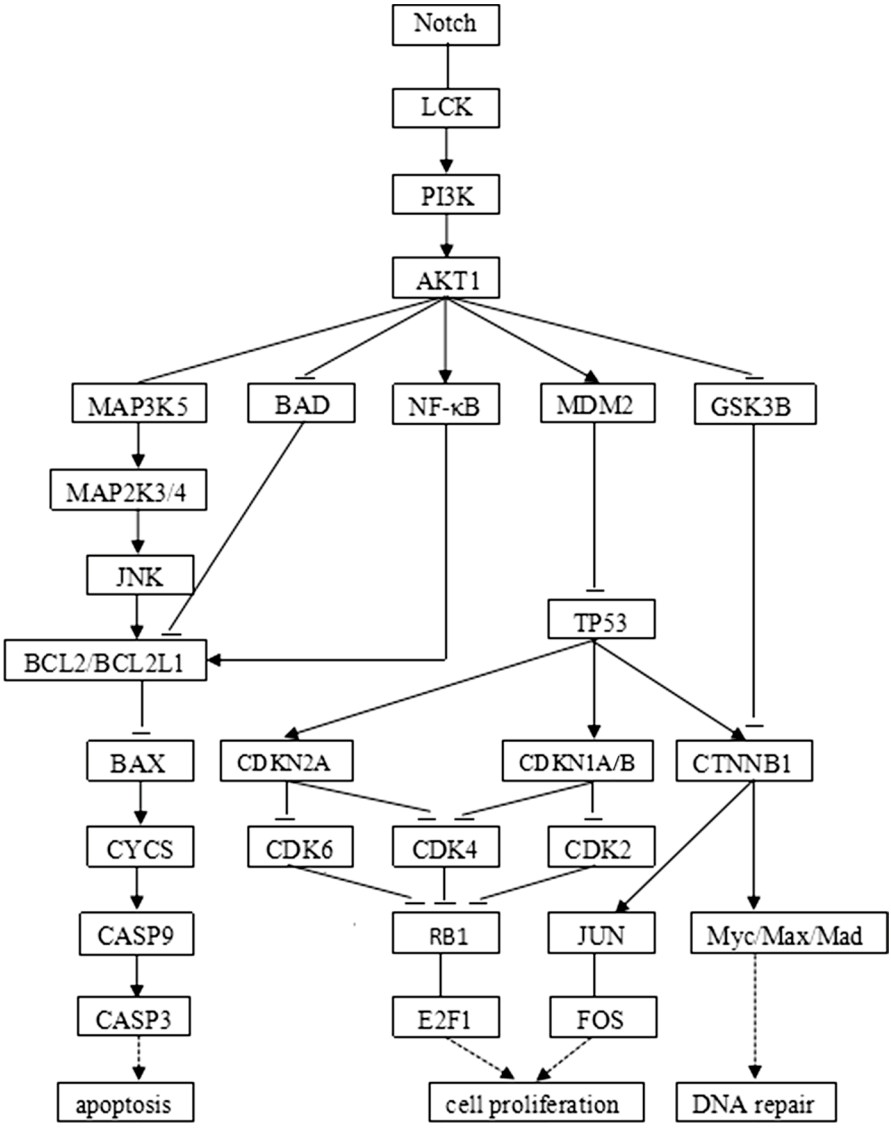

Fig. 3 The network of five major branches of Notch-mediated signaling pathway, which can regulate cell proliferation, apoptosis, and DNA repair. *branch 1* Notch/PI3K/Akt/MAPK (path 1), *branch 2* Notch/PI3K/Akt/Bad (path 2), *branch 3* Notch/PI3K/Akt/NF-κB (path 3), *branch 4* Notch/PI3K/Akt/P53 (paths 4, 5, 6, 7), *branch 5* Notch/PI3K/Akt/GSK3B (paths 8, 9) signaling pathways

Function Enrichment Analysis of Notch Signaling Pathway-Related Genes in 8 Liver Cell Types

EASE analysis software was employed for related genes sets of Notch signaling pathway, and the enriched GO categories were selected from each cluster according

Table 1 Comparative analysis of Notch-related genes which significantly change in rat 8 cell types of RL

Cell types	Recovery time points(h) after partial hepatectomy									Total
	2	6	12	24	30	36	72	120	168	
HC	11	4	10	10	11	7	*14*	5	7	25
BEC	16	19	*25*	21	16	8	4	15	7	38
HOC	5	*7*	4	2	4	5	2	5	2	15
HSC	*13*	8	*13*	8	4	5	6	7	8	25
SEC	8	*12*	6	6	*12*	6	6	8	9	24
KC	7	3	6	*10*	7	9	6	5	8	21
PC	8	12	4	9	12	10	*13*	10	5	27
DC	7	9	8	*15*	8	11	6	6	3	24

Italics fields represent the expression peaks in every cell type with most number of significantly changed genes

to the EASE scores (Wang et al. 2015). The results revealed that the Notch signaling-related genes were involved in cell proliferation, apoptosis, cell cycle progression, cell movement, cell response to stimulating factors, and so on in 8 liver cell types (Table 2).

To further clarify the physiological processes, the expression extremum of Notch signaling pathway-related genes was uploaded to the 'Dataset Files' of IPA. Then, core-analysis and comparison analyses were proceeded successively (Wang et al. 2016b). The heatmap of biofunctions in Fig. 5 indicated that Notch signaling pathway was related to cell proliferation, tumor cell lines proliferation, DNA repair, DNA synthesis and so on.

The Regulatory Role of Each Path of Notch Signaling Pathway in 8 Liver Cell Types

Genes of each path of Notch signaling pathway in 8 liver cell types involved in LR were picked up to clarify the exact mechanism of 9 paths of Notch signaling pathway in regulating the physiological activation of LR (Table 3). Besides, the expression extreme values of genes were entered into IPA software to "Core analysis" and "Comparison analysis," and to obtain the function cluster heat map of each path in 8 liver cell types (Yang et al. 2016) (Biofunction heatmap analysis of Notch-mediated nine signaling pathway-related genes in eight hepatic cell types by IPA software. Path 1, 2, 3). The results indicated that path 1 could promote the cell proliferation of tumor cell lines in almost the 8 liver cell types except SEC, which might be in line with the concept that over expression of N2ICD can result in the HCC formation. Besides, it is obvious that the cell viability of most eight live cell types increased at different extents in path 1. Path 2 could promote the cell migration and cell invasion of most cell types. The model of regulating cell physiological activities in path 3 was similar to path 2. Path 4 could promote the apoptosis of tumor cells in almost all cell types except HSC. Path 5 could promote the repair of DNA, and might be beneficial to cell proliferation of carcinoma cell lines except HSC and SEC. Path 6 could promote the repair of DNA, and inhibit apoptosis of BEC, HSC, and PC. Path 7,

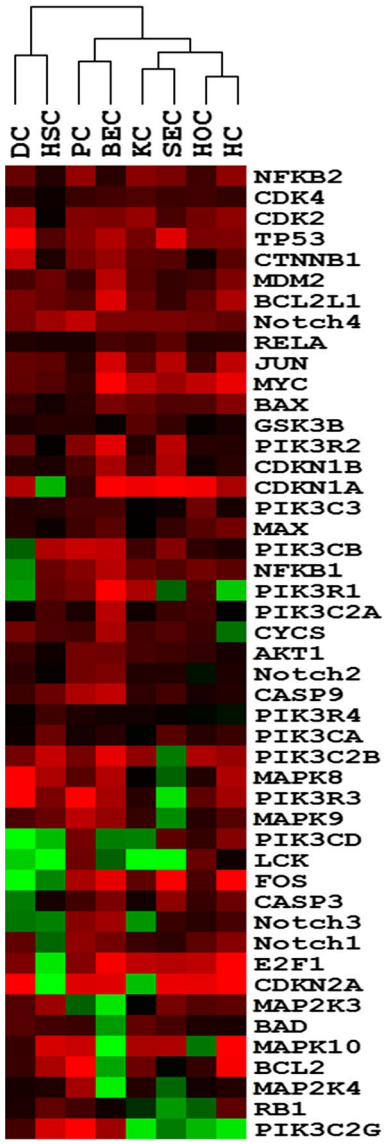

Fig. 4 Global expression profiles of Notch-mediated signaling pathway-related genes in eight liver cell types during rat liver regeneration. Red color denotes the expression levels higher than the control; however, green color is lower than the control (Color figure online)

Table 2 Overrepresented functional categories in five clusters of Notch-mediated signaling pathway-related genes during rat liver regeneration

Cluster enriched biological process	No. of genes	*P* value
Positive regulation of apoptotic process	12	9.20E−11
Regulation of cell cycle	9	1.80E−10
Intrinsic apoptotic signaling pathway in response to DNA damage	7	1.50E−09
G1/S transition of mitotic cell cycle	7	3.30E−09
Apoptotic process	10	6.80E−08
Response to toxic substance	7	2.00E−07
Cell cycle arrest	6	9.10E−07
Negative regulation of cell proliferation	9	1.30E−06
Regulation of apoptotic process	7	1.80E−06
Cellular response to UV	5	3.50E−06
Cellular response to hypoxia	6	1.20E−05
Negative regulation of mitotic cell cycle	4	4.60E−05
Cellular response to DNA damage stimulus	6	1.30E−04
NIK/NF-kappaB signaling	3	1.30E−04
Cell proliferation	6	1.40E−04
Negative regulation of cell growth	5	1.50E−04
Mitotic cell cycle arrest	3	4.20E−04
MAPK cascade	4	1.20E−03
Negative regulation of apoptotic signaling pathway	3	1.60E−03
Negative regulation of fibroblast proliferation	3	2.50E−03
Positive regulation of cell cycle	3	3.10E−03
Positive regulation of apoptotic signaling pathway	3	3.30E−03
Liver development	4	3.70E−03
Cell cycle	3	2.90E−02

path 8, and path 9 might be involved in promoting the transcription and the cell proliferation of tumor cell lines.

Discussion

After injury or PH, liver has a strong ability to recover its previous volume and function, which was involved in many physiological activities and regulatory pathways such as Hippo, NF-κB, and Notch signaling pathways. Notch, as a family (Notch1–4) of heterodimeric transmembrane receptors, is activated by ligands, and then the Notch intracellular domain (NICD) is cleaved and translocated to the nucleus where the downstream genes are activated, which is closely associated with a variety of fundamental cellular processes, including cell proliferation (Leong and Karsan 2006), differentiation (Huang et al. 2010), stem cell renewal and cell fate

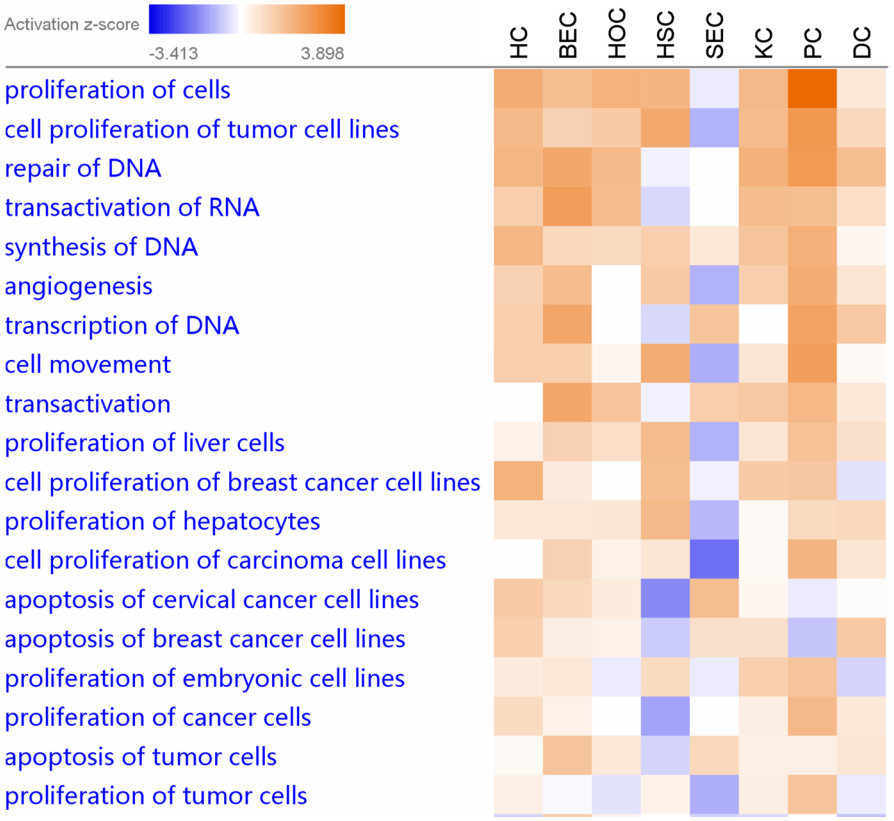

Fig. 5 Biofunction heatmap of Notch signaling pathway-related genes predicted by IPA software about eight liver cell types during rat liver regeneration. The color of heat map square represents the activity of biofunctions. Jacinth color represents activation of biofunction and blue represents inhibition of biofunction (Color figure online)

Table 3 Genes of each path of Notch signaling pathway that change significantly in 8 liver cell types

Signaling pathways	Cell types							
	HC	BEC	HOC	HSC	SEC	KC	PC	DC
Path 1	12	22	6	15	11	9	17	12
Path 2	12	21	6	14	9	10	15	12
Path 3	13	20	6	14	10	11	17	14
Path 4	11	18	9	15	12	11	16	12
Path 5	12	20	10	15	13	12	15	13
Path 6	11	19	6	13	11	9	16	13
Path 7	11	18	7	12	10	9	15	12
Path 8	9	17	5	12	10	8	15	12
Path 9	9	16	6	6	9	8	14	11
Total	25	38	15	25	24	21	27	24

decision (Wang et al. 2012a). Notch could function via PI3K/Akt/MAPK, PI3K/Akt/Bad, PI3K/Akt/NF-kB, PI3K/Akt/P53 signaling cascades (Fig. 3). Pajvani et al. had confirmed that Akt was the classic downstream path of the Notch signaling (Pajvani et al. 2013), inhibiting the activation of Akt; the downstream molecules activation of Notch signaling in hepatocytes could suppress hepatocyte proliferation and liver regeneration after PHx. Ping et al. (Ping et al. 2006) demonstrated that Notch/PI3K/Akt was important for the G1/S progression in hepatic SECs, which could induce the cytokine secretion and promote the SECs proliferation. Zhang et al. (Zhang et al. 2018a) provided direct evidence that inhibition of Notch pathway resulted in dysregulation of cell cycle and cell cycle components (CycinE1, A2, B1) in proliferating hepatocytes during LR. Wang et al. (Wang et al. 2012b) first noted that Notch-3 might play key roles for the generation of hepatocytes from fetal liver stem/progenitor cells (FLSPCs), which might serve as a new marker for FLSPCs. However, the actual roles and mechanisms of Notch signaling pathway in LR were obscure. To further investigate the exactly mechanism of Notch signaling pathway in eight liver cells types in LR, we separated and purified eight kinds of liver cells at 10 time points in regenerative liver after PH, checked the expression profiles of Notch-mediated signaling pathway-related genes in above eight liver cell types, and employed the IPA software to elaborate the physiological function of Notch in eight type cells in LR. As shown in Fig. 5, the physiological processes possibly regulated by Notch signaling pathway were almost the same in different cell types with exception of SECs

Several researches have confirmed that Notch could promote the proliferation of stem cell, T cell receptor-induced matured cells, and hepatocellular. In this study, the expression of anti-apoptosis genes Bcl2 was reinforced in most cell types at different levels. Figures 5 and 6 illustrate that cell proliferation, DNA repair, cell viability, cell movement in HCs, HOCs, BECs, and PCs were activated at different extents, suggesting that Notch might promote cell proliferation in rat regenerative livers. Tanimizu and Mitaka (Tanimizu and Mitaka 2014) noted that HOCs, as one kind of liver stem cells, will rapidly differentiate into HCs, BECs after liver was severely damaged or liver cell proliferation ability was restricted, which was partially consistent with the activities of Notch signaling pathway in HCs, HOCs, BECs, and PCs to defense liver injury. Previous studies found the Jagged1-Notch2 axis regulated biliary development from cholangiocytic commitment to final bile duct arborization, which was beneficial to liver regeneration (Sparks et al. 2010). Notch signaling pathway-related genes in BECs showed an expression peak at 12 h (Table 1). Furthermore, the number of up-regulated genes was more than that of down-regulated genes (Fig. 4), which was consistent with the result that liver-specific activation of Notch-2 could regulate normal biliary differentiation in mice to control liver development.

In Fig. 3, it was noticeable that the Notch/PI3K/AKT link was necessary in all 9 signaling pathways to mediate cell proliferation, apoptosis, and DNA repair, which agreed with the viewpoint of Veeraraghavalu et al. (2005) that the Notch/PI3K/AKT link was established to regulate the epithelial-mesenchymal transition to inhibit apoptosis in diverse cells. Besides, Sade et al. (Sade et al. 2004) also demonstrated the function of Notch-1 requires p56lck, PI3K-dependent and AKT/PKB-mediated anti-apoptosis

Fig. 6 Biofunction heatmap analysis of Notch-mediated nine signaling pathway-related genes in eight hepatic cell types by IPA software. *Paths 1, 2, 3* Notch/PI3K/Akt/MAPK, Notch/PI3K/Akt/Bad, and Notch/PI3K/Akt/NF-κB signaling pathway mediated anti-apoptosis activities, *paths 4, 5, 6, 7* Notch/PI3K/Akt/P53 signaling pathway modulated cell proliferation and DNA injury repair, *paths 8, 9* Notch/PI3K/Akt/GSK3B signaling pathway modulated DNA injury repair. The color of heat map square represents the activity of biofunctions. Red heatmap square means active biofunction, while blue heat map square means inhibitive biofunction at certain stage of liver regeneration (Color figure online)

signaling pathway in T cells. Previous studies had claimed Notch-1 signaling inhibited cell apoptosis via the cytoplasmic mitochondrial pathway. From Fig. 3, we could obviously found that Notch could inhibit cell apoptosis by paths 1 and 2, which was similar to the report that constitutive overexpression of the intracellular domain of Notch-1 (ICN1) promoted proliferation and suppressed apoptosis of stem cells by inhibiting cytochrome c release and caspase-9,-3 activation, accompanying with up-regulation of anti-apoptotic proteins Bcl-2 and down-regulation of pro-apoptotic proteins Bad and Bax(Chen et al. 2017). Thus, the cell proliferation and cell viability regulated by paths 1 and 2 were mostly activated in 8 liver cell types. Besides, from Fig. 4, we observed that the pro-apoptotic proteins Bad and Bax were barely expressed in 8 liver cell types. Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) also could cleave pro-apoptotic protein Bid to help release mitochondrial cytochrome c into the cytosol and then activate pro-caspase-9 and -3, which could induce apoptosis in cancer cell lines. However, Soderstrom et al. (Soderstrom et al. 2002) found that MAPK/ERK could suppress TRAIL-induced apoptosis by inhibiting initiator of caspase-9 activity, which was consistent with the path 1. Thomas et al. (Thomas et al. 2000) found that

the apoptosis of approximately two-third melanoma cells could be inhibited by overexpressing Bcl-2, which bound to the permeability transition pore in mitochondria to prevent cytochrome c release from mitochondria and change in MMP. Above all, Notch/PI3K/AKT/MAPK and Notch/PI3K/Akt/Bad pathway could inhibit cell apoptosis by cytoplasmic mitochondrial pathway. In summary, the Notch could inhibit cancer cell lines but promote the stem cell and tumor cell proliferation; the regulatory mechanisms revealed here may be related to liver regeneration, which was consistent with the results of Functional cluster analysis through IPA, which showed that paths 1 and 2 of Notch signaling pathway could promote tumor cell lines proliferation.

NF-κB, a downstream molecule of Notch/PI3K/Akt, was activated after PH to respond to cytokines and oxidative stress, which may play an important role in LR. Li et al. (2016) showed that the Notch-1/PP2A/PI3K/AKT1/NF-κB signaling pathway could promote cell invasion, migration, and adhesion in of human breast carcinoma MDA-MB-231 cell lines. Ramdass et al. (2007) had independently showed that Notch-1 could activate NF-κB activity through PI3K-PKB/AKT pathway to help human cervical cancer cell line-CaSki progression by increasing cell proliferation or blocking cell apoptosis through Bcl-2, which was in accordance with the results indicated in path 3 (Fig. 6) that the cell proliferation of tumor cell lines was promoted in almost 8 liver cell types. Besides, we also found that path 3 of Notch signaling pathway significantly promoted the cell proliferation, invasion, and migration, which was beneficial to the process of LR. In a word, the relationship between Notch-1/PI3K/AKT1/NF-κB and LR needs to be further illustrated.

P53, as a tumor suppressor, could integrate stress signals into a diverse of antiproliferative responses to activate apoptosis. From Fig. 3, it was evident that the cell cycle-related components were involved in PI3K/Akt/P53 signaling to regulate cell physiological activities. Blocking the Notch signaling pathway during liver regeneration may lead to delay in S phase entry, disturbance of S-phase and M-phase progress due to dysregulation of cell cycle components. Darwiche et al. (2011) found that inhibition of Notch during oval cell-mediated regeneration resulted in an aberrant hepatocellular mitoinhibition index (p21Waf1:Ki67) after regeneration. In path 4–9, the Notch signaling modulated cells proliferation or apoptosis with the activities of the cyclin-dependent kinases, which were consistent with above-mentioned literatures. The heatmap results showed that the paths 4, 5, and 6 of Notch/PI3K/AKT/P53 pathway could promote normal and tumor cell proliferation in the six types of liver cells except SECs and DCs, which were consistent with the above-mentioned literatures. Functional cluster analysis through IPA showed that Notch-P53 not only promoted cell proliferation but also inhibited cell proliferation from the networks of Notch signaling pathway. However, whether the Notch/PI3K/Akt/P53 could also promote liver cell proliferation requires further study.

Conclusion

In summary, Notch signaling pathway was closely related to rat liver regeneration. Genes related to Notch signaling pathway were significantly regulated in LR at different levels. In addition, functional enriched analysis indicated that Notch signaling

pathway might promote tumor cell lines proliferation, activation, and migration in eight liver cells. However, the conclusion is drawn mainly based on the microarray data and bioinformatics analysis, to further authenticate the role of Notch in liver regeneration remains further elaboration by using genes overexpression/knockout methods.

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Compliance with Ethical Standards

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

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