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HBV induces different responses of the hepatocytes and oval cells during HBV-related hepatic cirrhosis

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ABSTRACT

Although hepatitis B virus (HBV)-related cirrhosis and hepatocellular carcinoma (HCC) cause a sever health problem worldwide, the underlying mechanisms are still elusive. This study aimed to investigate the responses of different cell types isolated from HBV transgenic mice. A cross-sectional set of hepatocytes and oval cells were obtained from HBV transgenic and control mice. Flow cytometry, immunohistochemistry and microarray were applied to investigate the cell biology of the hepatocytes and oval cells. Our results showed that HBV induced the proliferation of both cell oval cells and hepatocytes, and induced cell death of HBV hepatocytes while had minimal effects on oval cells. Further molecular and pathways analysis identified some genes and signaling pathways may be responsible for the different responses between oval cells and hepatocytes. In addition, analyses of selectively ten genes by IHC staining in human samples were consistent with microarray data. In summary, HBV transgenic mice is a useful model for studying the biological behaviors of oval cells affected by HBV and HBV-cirrhosis. Also, our results help better understand the mechanisms of HBV induced cirrhosis, and provide novel progenitor markers or prognostic/therapeutic markers.

1. Introduction

Hepatitis B virus (HBV) infection is a major global health problem, which affects 257 million people worldwide. It has been reported that 75% of HBV-infected people are Asian while it only has a lower prevalence (0.3%–1.5%) in Western countries. Despite significant progress has been made over the past decades, HBV-induced liver cirrhosis still represents a major health burden, and the risk of developing hepatocellular carcinoma (HCC) significantly increases in patients with chronic hepatitis B and type B-related liver cirrhosis [1–4]. It is estimated that over 1.3 million deaths have been yearly reported from complications of liver cirrhosis, liver failure and HCC. Over 12% of patients with HBV cirrhosis die of liver failure, and up to 10% perish from HCC [5–8].

Although many studies have attempted to understand the physiological, cellular, and molecular mechanisms of the hepatitis diseases, the pathogenesis of which is not fully understood. Liver progenitor cells, named as oval cells in rodents, are thought to be localized in canals of Hering and exhibit bipotential differentiation into both hepatocytes and cholangiocytes [9,10]. Recent studies showed that expansion of oval cells was observed in human chronic liver diseases, including HBV cirrhosis [11–15]. And in HBV induced liver damage, activated immune response impaired the regeneration of hepatocytes regardless of hepatic progenitor cells [16,17]. In addition, a fibrogenic response and the severity of liver diseases was positively correlated with the activation of oval cells [18–21]. Furthermore, reports have documented that stem/progenitor cells might be the cell origins of HCC. Indeed, detailed immunophenotyping of human liver cancers revealed that they expressed progenitor cell markers [22,23]. Importantly, animal studies further showed that oval cells were involved in the

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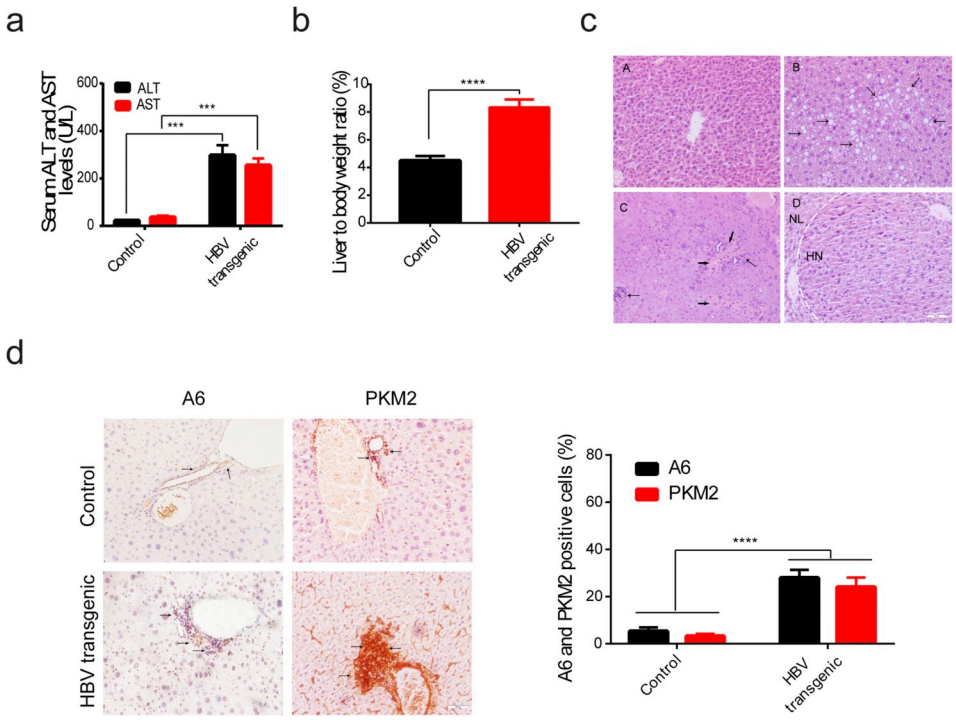

Fig. 1. HBV induced severe liver injury and oval cell proliferation. Compared with healthy controls, HBV transgenic mice had a significant higher level of both ALT and AST (**a**) and liver to body weight ratio (**b**). (**c**) HE staining showed that no histological changes were observed in livers of control mice (A); there were hepatic steatosis (B, arrow), fibrosis (C, thick arrow), inflammatory infiltration (C, thin arrow) and hyperplastic nodules (D) in the transgenic livers (H&E, × 100). NL: normal liver; HN: hyperplastic nodules. (**d**) Adult control mice were only positive for A6 in biliary cells and for PKM2 in few early hepatocytes. HBV accelerated oval cell response in the livers. A6-and PKM2-positive cell number was increased (× 200). Quantitation of A6-and PKM2-positive cells confirmed an increase in oval cell number. Data were represented from the replicate analysis as the mean ± SD. $^{*}p < 0.05$.

formation of liver tumors in rodent models [24–27] and the data from our lab also showed that hepatic progenitor cells might play a role in combined hepatocellular cholangiocarcinoma development [28]. Taken together, all these facts suggest that oval cells is tightly related to HBV associated diseases. However, few reports elucidate the role of oval cells in HBV transgenic animal models [29,30]. So identification of key genes and biological pathways responsible for oval cell activation induced by HBV infection will improve our understanding of oval cell biology and HBV-related cirrhosis and hepatocarcinogenesis.

The aim of the present study was to identify the characteristics of oval cells and hepatocytes and to analyze the gene expression patterns in these two types of cells isolated from HBV transgenic mice using a combination of large-scale microarray and sequencing strategies. Our results showed that HBV expression induced the proliferation of both cell types and the cell death of hepatocytes but with minimal effects on oval cells. Furthermore, we identified genes and signaling pathways differently displayed in oval cells and hepatocytes upon HBV infection. Our study help people better understand the response of oval cells and hepatocytes after HBV infection and give insights into how to target oval cells to battle against HBV related diseases.

2. Materials and methods

2.1. Materials and samples

8-week male HBV transgenic BALB/c mice (n = 20) were obtained from PLA Liver Center (No. 458 Hospital of PLA, Guangzhou, China) and control mice (n = 20) were obtained from the Laboratory Animal Center of Tongji Medical College (Huazhong University of Science and Technology, China). HBV transgenic mice were generated as previously reported [31]. Briefly, the HBV DNA containing 1.3 times of HBV genome obtained from pUC19-HBV(C)1.3 plasmid following Sma I digestion was micro-injected into blastocysts of female BALB/c mice. The female transgenic mice were crossed with male wild type mice and the offspring HBV mice were used in this study. The animals were housed under pathogen-free conditions. Rat anti-A6 antibody was a generous gift from Dr. Valentina M Factor (National Cancer Institute, National Institutes of Health, Bethesda, USA). Kinase inhibitors, Assay Kit and other chemicals as well as the antibodies were listed in Sup Tables 1 and 2

2.2. Microarray analysis

Primary hepatocytes and oval cells isolated from normal and HBV transgenic mice were lysed in Trizol reagent and the mRNA were isolated and purified. Reverse transcription was performed using the Ambion® Illumina TotalPrep RNA Amplification Kit according to the manufacturer's protocol. Labelled cDNAs were hybridized and dispensed onto Illumina Mouse WG-6 v2.0 Expression BeadChips, which contains 45,281 genes. The arrays were read and imaged using the Illumina BeadArray Reader … A quality control analysis was performed within the Illumina BeadStudio software to eliminate the systematic variation of non-biologic origin within the raw microarray data using the Cubic Spline method. The significant E-dependent differentially expressed genes were obtained from comparison of HBV transgenic cells to the control cells and were defined by Diffscore > 20.0 and a P-value cut-off of 0.05 (derived from ANOVA). Microarray data was uploaded to the NCBI database with GEO number as GSE39289.

2.3. Statistical analysis

Data are presented as the mean ± SEM, and analyzed statistically using the Student's t-test, ANOVA or the $\chi 2$ test as appropriate. A p value < 0.05 was considered to be statistically significant.

Other detailed methods were listed in supplementary materials and methods.

3. Results

3.1. Estimation of HBV profile in sera

To confirm the genotype of HBV transgenic mice, the HBV mRNA and the associated antigen proteins were analyzed. The data from RT-PCR showed that HBV mRNA was expressed in transgenic mice (the

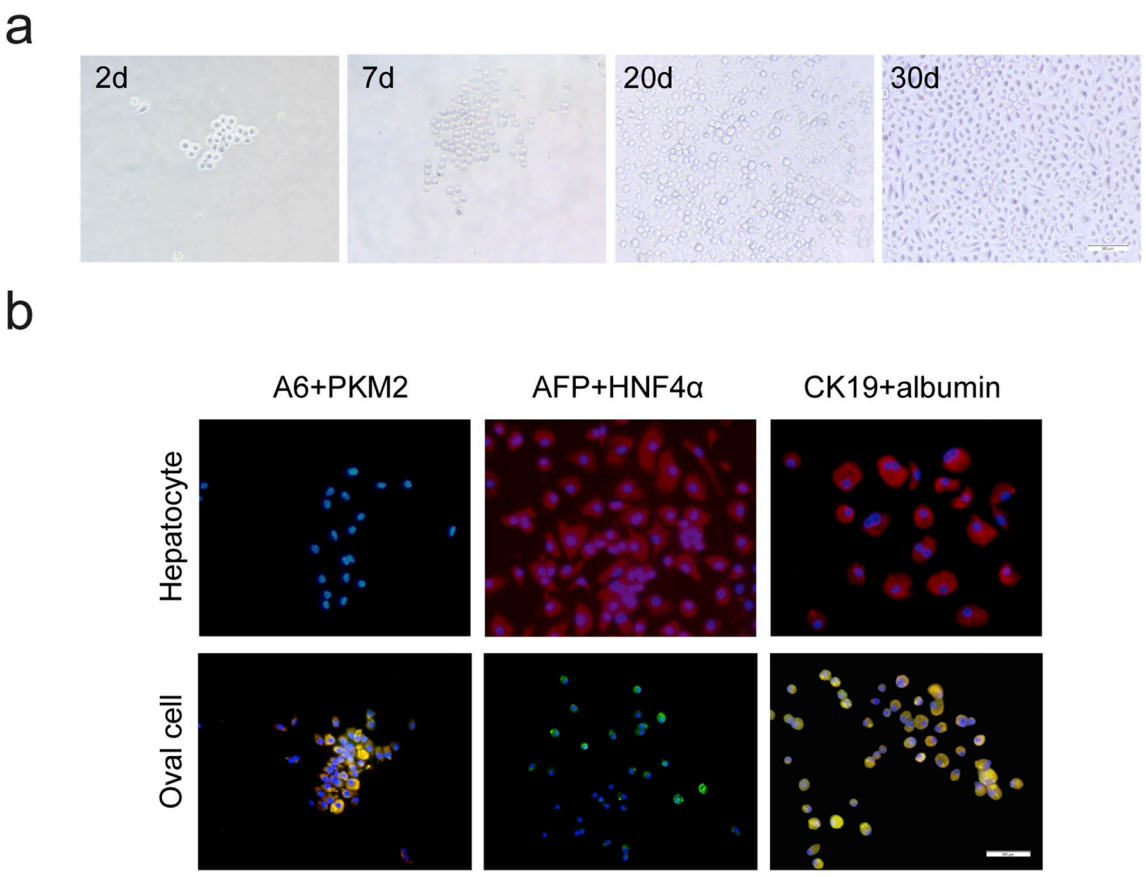

Fig. 2. Primary culture and characterization of oval cells. (**a**) Phase-contrast morphology of cultured oval cells. Original magnifications, × 100. (**b**) The confocal IF analysis showed that primary cultured hepatocytes were negative for A6 (green), PKM2 (red), AFP(green), and CK19(green), and positive for HNF4α(red) and albumin (red). And primary cultured oval cells were positive for A6 (green), PKM2 (red), AFP(green), CK19(green) and albumin (red), and negative for HNF-4α(red). Hochest 33258 was used to stain for nuclei (blue). Original magnifications, × 200.

data were provided by PLA Liver Center, data not shown). Further, HBsAg, HBsAb, HBeAg, HBeAb and HBcAb were qualitatively assessed in the Dept. of Clinical Laboratory of Tongji Hospital. In HBV transgenic mice, the detection of HBsAg and HBeAg was positive while negative results were obtained using these three antibodies:HBsAb, HBeAb and HBcAb. The concentration of HBsAg was 250 IU/ml (normal: < 0.5 IU/ml) and the concentration of HBeAg was 1.66 S/CO (normal: < 1.0 S/CO). The results were consistent with the other previous studies [32,33].

3.2. HBV transgenic mice show low survival rates and increased liver damage

Although transgenic mice are not the natural host of HBV, increased mortality was observed in HBV transgenic mice (60%; 12/20 *vs.* 0%; 0/20 in control). To assess liver injury in HBV transgenic mice, we measured serum ALT and AST levels, which were markedly elevated in HBV transgenic mice compared to those in control cohorts (Fig. 1(a)). The ratio of liver to body weight in HBV mice was also higher than that in control (Fig. 1(b)). No histological alterations were observed in livers of control mice (Fig. 1 (c).A). However, we clearly observed ballooning of the hepatocytes and cytoplasmic vacuoles in the transgenic livers. In addition, fibrosis, immune cell infiltration and increased mitosis were also frequently observed in HBV transgenic livers (Fig. 1 (c).B-C). Furthermore, hyperplastic nodules were present in some HBV transgenic mice (Fig. 1 (c).D).

3.3. Oval cells amplification in HBV transgenic mice

Oval cell activation was frequently observed in human cirrhotic samples [11–15]. To confirm whether HBV expands oval cell population in mice, we performed immune-histochemical staining for two well-known oval cell markers: A6 and PKM2. PKM2 was expressed in the oval cell and early hepatocyte, while A6 was in the oval cell and biliary tract. Analysis of liver sections from healthy control mice revealed that few oval cells were noted in the livers, where A6 was stained in biliary cells and PKM2 was stained in few early hepatocytes (Fig. 1 (d)). However, the number of oval cells was increased in the transgenic mice, as A6 and PKM2-positive cells were frequently observed in the periportal zone and in the vicinity of the portal tracts (Fig. 1 (d)). These cells were organized into clusters, with a few cells forming primitive ductular structures with a poorly defined lumen. Quantification of A6 and PKM2-positive cells exhibited that oval cell amplification existed in HBV transgenic mice (Fig. 1 (e)).

3.4. Primary culture and characterization of oval cells and hepatocytes

Oval cells isolated from control mice adhered to plates within 2 days, and started to proliferate at day 7 (Fig. 2 (a)). In contrast, primary oval cells isolated from HBV mice proliferated much more quickly than controls, with a half shorter population doubling time (data not shown).

Double immunofluorescence staining was performed to characterize the primary oval cells and hepatocytes. The results showed that oval

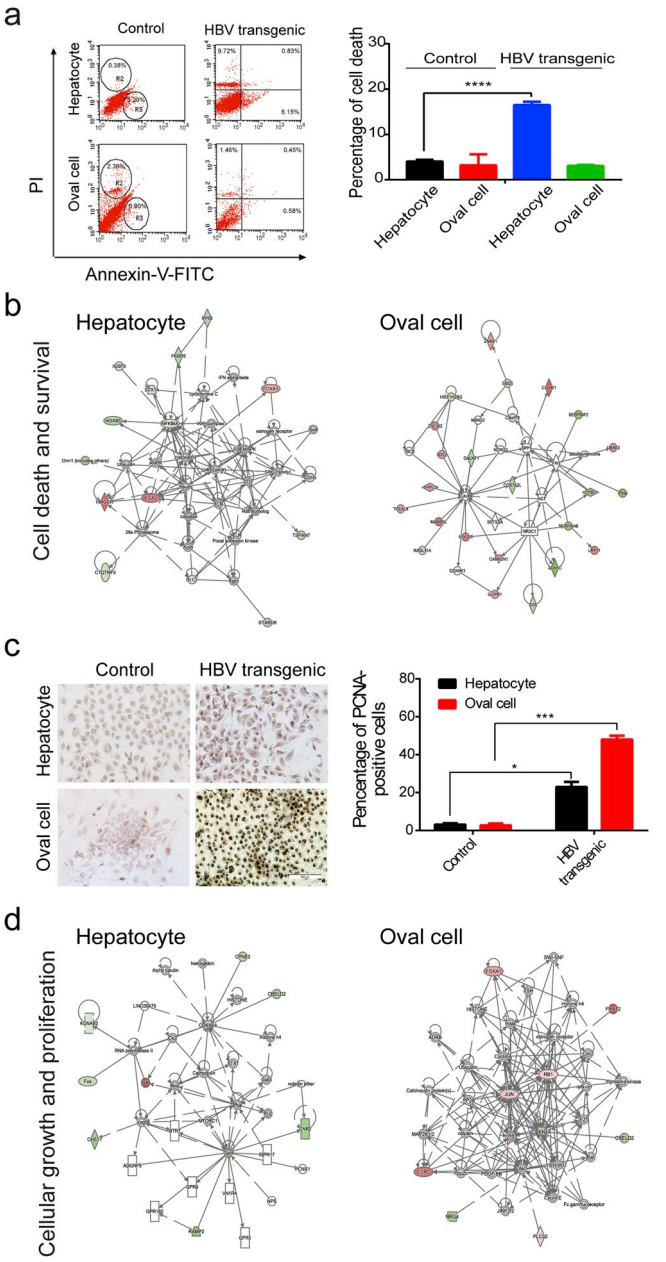

Fig. 3. HBV had different effects on cell death and cell proliferation between hepatocytes and oval cells. (**a**) Flow cytometric analysis was used to measure annexin-V-FITC/PI-positive cells. The statistical analysis of the data showed that HBV had a significant effect on cell death of the hepatocytes but had little effect on those of oval cells. (**b**) Cell death and survival genes in hepatocytes and oval cells; (**c**) PCNA staining showed that there was significant higher PCNA-positive ratio in HBV transgenic oval cells that that in HBV transgenic hepatocytes. (d) Cellular growth and proliferation associated genes in hepatocytes and oval cells affected by HBV. Data were represented from the replicate analysis as the mean ± SD. $^*p < 0.05$.

cells expressed A6, PKM2, AFP, CK19 and albumin, but were negative for HNF-4α. However, cultured hepatocytes were negative for A6, PKM2, AFP, and CK19. Albumin and HNF-4α were positively detected in these primary cultured hepatocytes (Fig. 2 (b)).

3.5. HBV causes an imbalance in apoptosis and cell proliferation

As shown in Fig. 3 (a), the number of annexin V and/or PI-positive hepatocytes was significantly increased in HBV transgenic mice compared with the controls, suggesting that early apoptosis and necrosis were the predominant mechanisms accounting for cell death in hepatocytes induced by HBV. On the contrary, oval cells from both control and HBV transgenic mice underwent very low levels of cell death (Fig. 3 (a)). Furthermore, PCNA staining in Fig. 3 (c) revealed that HBV had better capacity to promote cell proliferation of oval cells (69.97%) than that of hepatocytes (21.32%). Collectively, we postulated that higher proliferation and slight cell death of oval cells allowed them to persist for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development.

We further analyzed the related genes involved in cell death and proliferation. The data showed that 15 out of 23 and 24 out of 28 cell death related genes were deregulated in hepatocytes and oval cells, respectively. As to proliferation, 11 out of 34 and 10 out of 35 associated genes were altered in hepatocytes and oval cells, respectively. Detailed changes of these gene expression and the accompanying relations were shown in Fig. 3 (b) and (d). These data suggested different genes were involved in HBV-induced cell death and proliferation in these two different cells.

3.6. HBV causes different transcriptional responses in oval cells and hepatocytes

In gene expression analyses, 31,394 probes gave detectable signals. Cubic Spline normalization was used according to Illumina BeadStudio Gene Expression Module protocol. Compared with the controls, a total of 403 distinct genes were identified in hepatocytes from HBV transgenic mice with a Diffscore > 20.0 and fold change > 2. Of these, 123 were up-regulated and 280 were down-regulated (Fig. 4, Sup. Table 4). Comparison of the gene profiles in oval cells isolated from HBV transgenic mice and control mice showed that 1732 genes were differentially expressed. Of these, 728 were up-regulated and 1004 were down-regulated. Interestingly, only 36 up-regulated and 60 down-regulated genes were overlapped in both cell types (Fig. 4), indicating they responded differently to HBV at the molecular level.

To gain evidence for possible altered biological processes and signaling pathways, the identified genes was subjected to analysis using the Ingenuity Pathways Analysis (IPA) database (http://www.ingenuity.com), which were shown in Sup. Table 5. Among 403 identified genes in hepatocytes, 232 genes were mapped to the database (65 up-regulated and 167 down-regulated). Network analysis of these genes (Sup Figs. 2–5) showed 18 networks modules across four major networks were identified (Fig. 4): a) Cell-medicated immune response, cellular movement, cellular function and maintenance (Network Score 71, Sup. Fig. 3(a)); b) Cellular growth, development, tissue development, (Network Score 44, Sup. Fig. 2 (a), Sup. Fig. 4(a)); c) cellular morphology, cellular assembly and organization (Network Score 33, Sup. Fig. 5(a)), d) cell death and survival (Network Score 37, Sup. Fig. 2 (c)). Overall, genes in this group were associated with the following diseases and biological processes: cancer, inflammatory disease, hepatocellular carcinoma, hepatic fibrosis, liver hyperplasis/hyperproliferation, liver damage, and liver cirrhosis.

For these 1732 altered genes in oval cells, 926 genes were mapped to IPA database (315 upregulated and 611 down-regulated genes). Thirty modules across four major networks were identified (Fig. 4): a) Antimicrobial response, inflammatory response (Network Score 48, Sup Fig. 3 (b)), b) Cell death and survival (Network Score 37, Sup. Fig. 2 (d)); c) Cell growth, cellular movement (Network Score 50, Sup. Fig. 2 (b)); d) cellular assembly and organization (Network Score 25, Sup Fig. 5(b)); e) cell cycle and DNA replication, recombination, and repair pathways (Network Score 52, Sup Fig. 6 (a)(b)). Genes in this group were associated with the following diseases and biological processes: Organismal injury and abnormalities, Hepatic system disease, increased levels of ALT/AST/LDH/Hematocrit, liver steatosis, liver proliferation, liver inflammation/Hepatitis, liver damage, and liver cholestatsis.

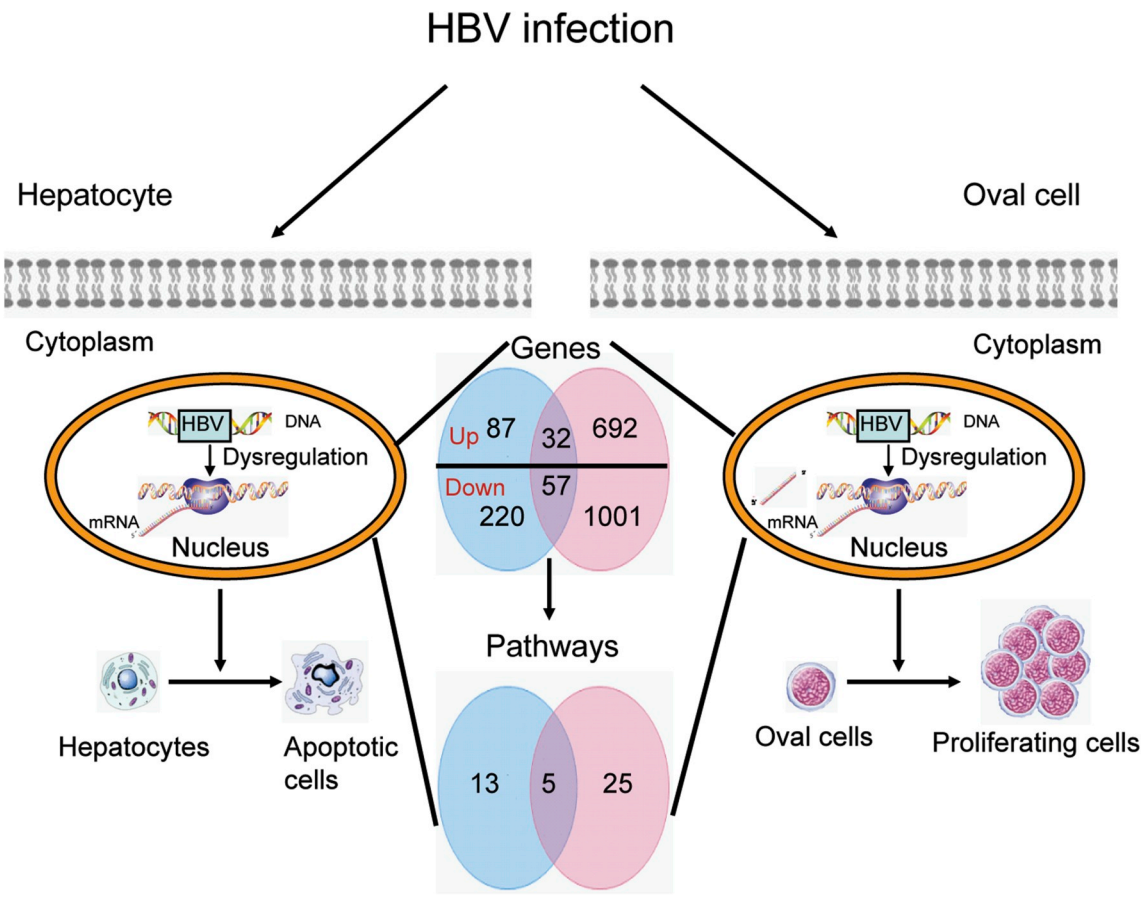

Fig. 4. Schematic illustration of the different effects of HBV on hepatocytes and oval cells. HBV induced deregulation of less genes and pathways in hepatocytes but affected more genes and pathways in oval cells. And HBV failed to affect cell death but induced cell proliferation of oval cells.

Table 1
Oncogenes and tumor suppressor affected by HBV.

	Hepatocyte	Oval cell
UEO	(n = 5): c-Fos*, c-Jun*, FDPS, Crk1,Fgfr1op2	(n = 22): ets2, FAT10, Magee1, LAPTM5, LMO2, MCL-1, c-Fos*, c-Jun*, RBM17, rras2, Snai2, Spy, mafg, rab39, rab8b, Fosb, maff, rap2c, Nr2f1, Il6, Il10, Aff1
DEO	(n = 14): p8*, PECI*, RelB*, Akt3, bcl-3, HGF, maf, NF-kappa B, Id1, IGF2, LAPTM5, rab27a, vav1, rgl2	(n = 22): p8*, PECI*, RelB*, Bad, BRE, CCT3, erbB1, ets1, park7, rab8a, mcm3, MRPS11, h-ras1, HSPCB, Gsto1, sas, Ruvbl2, rab13, rab1b, rab4a, rab5a, rab7
UET	(n = 7): Rb*, TTP*, RhoE, DSCR1, DUSP6, FHIT, smad3	(n = 7): Rb*, TTP*, PDCD7, Spred1, WT1, RhoB, Notch1
DET	(n = 8): DLC1*, IGFBP3*, p21*, DKK3, TGF-β1, IGFBP7, Notch1, RhoB	(n = 12): DLC1*, IGFBP3*, P21*, RhoE, CEACAM1, COPEB, TIP30, DDB1, Drg1, Drg2, ku70, FGL1

Abbreviation: UEO: up-expressed oncogene; DEO: down-expressed oncogene; UET: up-expressed tumor suppressor gene; DET: down-expressed tumor suppressor gene.
*: selected genes co-expressed in both hepatocyte and Oval cell, and verified with qRT-PCR.

3.7. Identification of oncogenes and tumor suppressor genes deregulated by HBV expression

Although failure of HCC formation was found in transgenic mice, hyperplastic nodules and precancerous lesion of HCC were observed in transgenic mice. Therefore, we analyzed gene expression levels of oncogenes and tumor suppressor in hepatocytes and oval cells. 344 oncogenes and 115 tumor suppressors from a comprehensive list obtained from several websites: http://www.ncbi.nlm.nih.gov/sites/entrez/, http://www.sabiosciences.com/and http://www.genome.jp/kegg/ were selected to investigate. Table 1 displayed the dysregulated oncogenes and tumor suppressors affected by HBV. Interestingly, we found that the number of down-regulated oncogenes (n = 14) was greater than that of up-regulated ones (n = 5), while the number of down and up-regulated tumor suppressor genes was similar (n = 8 and n = 7, separately) in hepatocytes. In oval cells, HBV induced dysregulation of cancer-related genes to a greater extent: although the number of up-regulated oncogenes was same as that of down-regulated oncogenes (n = 22), HBV suppressed the expression of many tumor suppressors (Table 1). In order to confirm the accuracy of the data, we compared our results to previously published literatures (Sup. References), in which genes were deregulated in HBV-related HCC or HCC caused by other factors. Of note, a consistence was gotten from the comparison in Table 2A and Table 2B.

According to this exhaustive literature search, ten of genes from Table 1 (IGFBP3, PECI, RelB, TTP, c-Fos, Rb, p21, DLC1, c-Jun, and p8) were selected to validate by qRT-PCR (primer were listed in Sup. Table 2), which displayed a good correlation between these two methods (Sup. Fig. 1).

Table 2A
Deregulation of tumor-related genes in hepatocytes.

Hepatocyte				Hepatocyte			
oncogene	Regulation	Reported results	Diseases	Tumor suppressor gene	Regulation	Reported results	Diseases
c-Fos	Up	Up	HBV[1], HBV-HCC[2]	RhoE	Up	Up	HBV-HCC[25]
c-Jun	Up	Up	HBV[1], HBV-HCC[2]	DSCR1	Up	Up	Lung cancer[26]
FDPS	Up	Up	HBV[3],HBV-HCC[4]	DUSP6	Up	Up	HBV, HBV-HCC[27]
Crk1	Up	Up	HCC[5]	FHIT	Up	Up	HCC[28]
Fgfr1op2	Up	Up	HCC[6]	Rb	Up	Up	HBV, HBV-HCC[29]
Akt3	Down	Up	HCC[7]	Smad3	Up	Up	HBV[30], HCC[31]
Bcl-3	Down	Up	HBV[8], HCC[9]	TTP	Up	Up	HBV[32], HCC[33]
PECI	Down	Down	Prostate cancer[10]				
p8	Down	Down	HBV, HBV-HCC[11]				
HGF	Down	Up	HBV[12], HBV-HCC[13]	DKK3	Down	Down	HCC[34]
MAF	Down	Up	HCC[14]	DLC1	Down	Down	HBV-HCC[35]
NF-kappa B	Down	Up	HBV,HBV-HCC[15]	IGFBP3	Down	Down	HBV[36], HBV-HCC[37]
Id1	Down	Up	HBV,HBV-HCC[16]	TGF-β1	Down	Down	HBV, HBV-HCC[38]
IGF2	Down	Up	HBV,HBV-HCC[17]	IGFBP7	Down	Down	HCC[39]
LAPTM5	Down	Up	HCC[18]	p21	Down	Down	HBV[40], HBV-HCC[41]
RelB	Down	Down	HBV[19],HCC[20]	RhoB	Down	Down	HBV, HCC[42]
rab27a	Down	Up	HBV[21], HCC[22]	Notch1	Down	Down	HBV, HCC[43]
Vav1	Down	Up	HBV,HBV-HCC[23]				
rgl2	Down	Up	Pancreatic cancer[24]				

Note: All references listed in this table are Sup References. Italic and bold names of genes mean that expression of these genes is contrary to the data of literatures.
Abbreviation: HBV: positive Hepatitis B virus; HCC: hepatocellular carcinoma.

Table 2B
Deregulation of tumor-related genes in oval cells.

Oval cell				Oval cell			
Oncogene	Regulation	Reported results	Diseases	Oncogene	Regulation	Reported results	Diseases
c-Fos	Up	Up	HBV[1], HBV-HCC[2]	***Bad***	Down	Up	HBV[70], HBV-HCC[71]
c-Jun	Up	Up	HBV[1], HBV-HCC[2]	***BRE***	Down	Up	HCC[72]
ets2	Up	Up	HBV[44], HCC[45]	***CCT3***	Down	Up	HCC[73]
FAT10	Up	Up	HBV[46], HBV-HCC[47]	***erbB1***	Down	Up	HCC[74]
Magee1	Up	Up	Breast cancer[48]	***ets1***	Down	Up	HBV[75], HCC[76]
LAPTM5	Up	Up	HCC[18]	park7	Down	Down	HBV-HCC[77]
LMO2	Up	Up	Lung cancer, teratocarcinoma[49]	***rab8a***	Down	Up	HBV-HCC[78]
MCL-1	Up	Up	HBV[50], HBV-HCC[51]	p8	Down	Down	HBV, HBV-HCC[11]
RBM17	Up	Up	HCC[52]	PECI	Down	Down	Prostate cancer[10]
rras2	Up	Up	HCC[53]	RelB	Down	Down	HBV[19],HCC[20]
Snai2	Up	Up	HCC[54]	***mcm3***	Down	Up	HCC[79]
Spy	Up	Up	Krebs-2 carcinoma[55]	MRPS11	Down	Down	HCC[80]
mafg	Up	Up	HBV, HCC[56]	***h-ras1***	Down	Up	Liver tummor[81]
rab39	Up	Up	Many types[57]	***HSPCB***	Down	Up	HBV[82], HCC[4]
rab8b	Up	Up	Testicular cancer[58]	Gsto1	Down	Down	HBV-HCC[83]
Fosb	Up	Up	HBV[59], HBV-HCC[60]	***sas***	Down	Up	HCC[84]
maff	Up	Up	Breast cancer[61]	***Ruvbl2***	Down	Up	HCC[85]
rap2c	Up	Up	colorectal cancer[62]	***rab13***	Down	Up	HCC[86]
Nr2f1	Up	Up	HBV[63], HCC[64]	***rab1b***	Down	Up	HCC[87]
Il6	Up	Up	HBV[65], HBV-HCC[66]	***rab4a***	Down	Up	Breast cancer[99]
Il10	Up	Up	HBV[67], HBV-HCC[68]	***rab5a***	Down	Up	Breast cancer[88]
Aff1	Up	Up	HCC[69]	rab7	Down	Down	Many types[89]

Tumor suppressor gene				Tumor suppressor gene			
Rb	Up	Up	HBV, HBV-HCC[29]	DLC1	Down	Down	HBV-HCC[35]
TTP	Up	Up	HBV[32], HCC[33]	IGFBP3	Down	Down	HBV[36], HBV-HCC[37]
PDCD7*	Up	Up	acute myeloid leukemia[90]	p21	Down	Down	HBV[40], HBV-HCC[41]
Spred1	Up	Up	HCC[91]	CEACAM1	Down	Down	HBV, HBV-HCC[93]
WT1*	Up	Up	HCC[92]	COPEB	Down	Down	HCC[94]
RhoB	Up	Down	HBV, HCC[42]	TIP30	Down	Down	HBV, HBV-HCC[95]
Notch1	Up	Down	HBV, HCC[43]	DDB1	Down	Down	HBV[96], HCC[97]
				Drg1	Down	Down	Colon cancer[98]
				Drg2	Down	Down	HBV, HBV-HCC[99]
				ku70	Down	Down	HBV[100], HCC[101]
				FGL1	Down	Down	HCC[102]
				RhoE	Up	Down	HBV-HCC[25]

Note: All references listed in this table are Sup References. Italic and bold names of genes mean that expression of these genes is contrary to the data of literatures. * Up-regulations of genes works as oncogenes.
Abbreviation: HBV: positive Hepatitis B virus; HCC: hepatocellular carcinoma.

Table 3
Preoperative clinical data for three groups.

Variable	Normal group	HBV group	HBV + HCC group	*p* value
Case	10	10	36	
Age	43.1 ± 13.93	42.5 ± 9.63	45.4 ± 8.39	0.203
Sex (M/F)	4/6	6/4	23/13	0.002
ALT (U/L)	10.2 ± 3.19	24.6 ± 11.06	55.6 ± 31.66	0.042
AST (U/L)	17.4 ± 3.03	23.3 ± 7.76	53.1 ± 22.72	0.001
AFP (ng/ml)	2.93 ± 1.32	2.95 ± 2.57	8486.9 ± 3133.51	0.519

Abbreviation: HBV: positive Hepatitis B virus; HCC: hepatocellular carcinoma; ALT: alanine aminotransferase.
AST: Aspartate Aminotransferase; AFP: alpha-fetoprotein.

3.8. Expression of selected genes in HBV positive patients

To further validate the results from microarray analysis, immunohistochemistry (IHC) was performed on 56 human samples including 10 normal controls, 10 HBV without HCC, and 36 HBV with HCC. Patients' preoperative clinical and laboratory data were shown in Table 3. We verified these 10 co-expressed genes based on the intersection of up/down-regulated genes list from Table 1. Staining results of these 10 genes were shown in Fig. 5. The positive rates of selected genes in human samples were listed in Table 4. Statistical analyses of the relative grayscale values and the positive rates showed that changes in the protein expression of the selected genes were consistent with the changes observed in mice models.

4. Discussion

Using HBV transgenic mice, Kim, et al. first reported that the HBx gene caused HCC [34]. Since then, transgenic mice have become a useful experimental model for defining the molecular events of HBV hepatitis, and HBV-related hepatocarcinogenesis. In the present study, we first confirmed viral persistence in the mice, which was reflected by positive expression of HBV mRNA and viral proteins (HBsAg+, HBeAg+). Chronic HBV infection in patients is associated with chronic hepatitis and liver fibrosis, which is characterized by leukocyte infiltration and collagen deposition in portal/periportal regions of the liver [35]. Attack towards liver by HBV virus will lead to its damage. Fibrosis represents early damage in the liver while cirrhosis is resulted from severe damage with scar in the liver tissue. In this model, liver injury and degeneration, including elevated serum ALT and AST levels, ballooning of hepatocytes, fatty changes, fibrosis, inflammation and hyperplastic nodules were noticeable in the late stage of HBV-mediated liver pathogenesis (around 10 months of age). Importantly, we also observed different responses of oval cells and hepatocytes upon HBV infections at the cellular and molecular level. To the best of our knowledge, this is the first report to identify oval cell activation induced by HBV expression in rodent model. We believe oval cells activation may be involved in HBV induced pathogenesis and carcinogenesis.

The HBV x protein (HBx) is involved in numerous biological progresses by either interacting with cellular proteins or serving as a coactivator for various transcription factors [36]. For example, HBx is preferentially recruited to the promoters or distal enhancers of certain genes which contain consensus binding sites for NFκB, AP1 and CREB [36]. Here, we found that HBV had minimal effects on the apoptosis of oval cells while induced higher levels of cell death of hepatocytes. On the contrary, a higher proliferation rate was observed in oval cells compared to hepatocytes in HBV transgene mice. The possible explanation is that the HBx binding transcription factors are differentially expressed between oval cells and hepatocytes so that the participation of HBx into transcriptional regulation or signaling transduction varies in these two types of cells. In addition, HBV DNA integration into genome also causes dysregulation of gene expression which is attributable to the interruption of gene transcription resulted from the insertion of HBV DNA into the active transcriptional sites. The difference on the accessibility of HBV DNA into the chromatin between oval cells and normal hepatocytes probably results in different molecular responses, leading to the differential response of oval cells and hepatocytes upon HBV infection. The active response of oval cells indicates that they can survive from HBV-induced injury and persist for a sufficient length of time to acquire genetic alterations for neoplastic development.

HBV related diseases is a complex disease with a multi-step process from preneoplastic lesions. Although we failed to detect the HCC in this model, precancerous lesion and hyperplastic nodules were present in livers expressing HBV. So we deemed this model is a valuable tool to explore the underlying molecular mechanisms of HBV-induced hepatitis, cirrhosis and liver tumors. To our knowledge, the molecular pathogenesis of HCC development resulted from simple investigation of liver tissues in rodent models and few genetic alterations within various cell types were explored in the liver upon HBV infection, mainly because of difficulties associated with the isolation and culture of primary adult oval cells. In this manuscript, we successfully isolated and cultured primary adult oval cells from both HBV transgenic mice and healthy controls, which guaranteed us to explore the molecular changes of oval cells after HBV infection. Interestingly, we found there is differential regulation of certain genes between oval cells and hepatocytes in response to HBV infection, suggesting some genes may serve as new progenitor markers or therapeutic targets for oval cells. For instance, our data showed that the TNF, EGF, IFNα, IL6, PPAR, and TGF-β signal pathways were deregulated in HBV-infected oval cells, which was consistent with the results from the previous study [37]. Therefore, inhibitors specific to these pathways may be an ideal treatments for HCC development.

Furthermore, we also found that both activation of oncogenes and inactivation of tumor suppressor genes occurred in HBV oval cells, which showed opposite pattern in HBV hepatocytes. These genes might take part in neoplastic transformation of oval cells induced by HBV regarding the multipotent role of oval cells. Given the regulatory elements of evolutionarily related species are conserved, the gene expression signatures reflecting similar phenotypes in different species may also be conserved [38]. This hypothesis is supported by numerous studies showing that comparison of gene expression data from human HCC and rodent HCC models can identify conserved molecular pathways responsible for aberrant phenotypes [39–41]. Indeed, staining results from human samples were consistent with microarray data from HBV mice, suggesting molecules and signaling pathways identified in this model help us understand the biology and carcinogenesis of HBV related diseases.

Collectively, our data have demonstrated that HBV could trigger differential responses in hepatocytes and oval cells. The molecules and signaling pathways identified in HBV transgenic oval cells may be involved in their activation and malignant transformation. Therefore, it is promising to develop these genes as progenitor markers and therapeutic targets for HBV related hepatocarcinogenesis. Our data also facilitates further research into the molecular mechanisms of proliferation and transformation of oval cells induced by HBV.

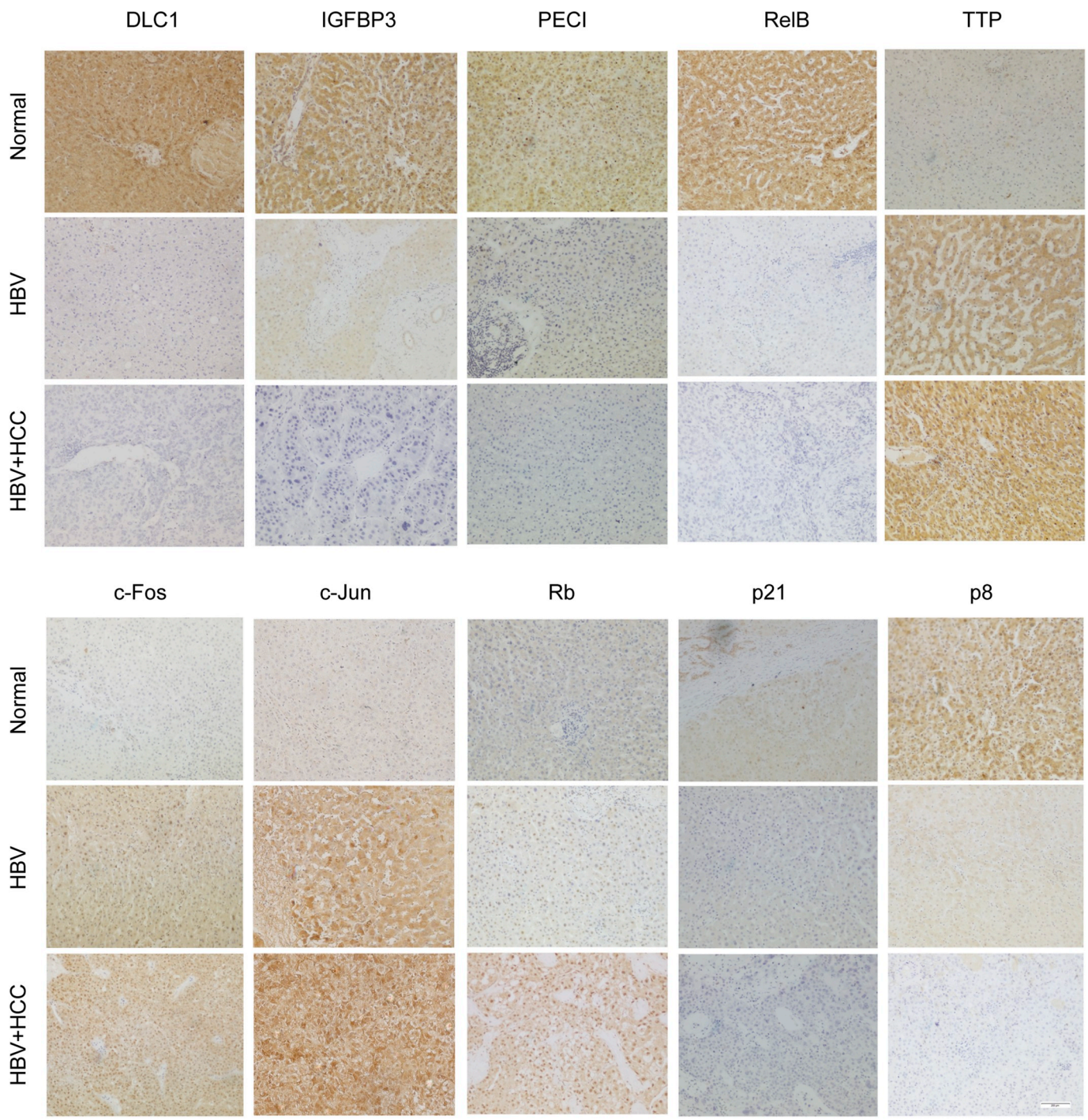

Fig. 5. Histological analysis of 10 selected genes in human samples. The expression levels were determined by immunohistochemistry using sections from the healthy controls, patients with HBV infection, patients with HBV induced HCC. Specific signals were represented by brown staining. Original magnifications, × 100.

Table 4
The positive rates of selected gene in human samples.

Groups	IGFBP3	PECI	RelB	TTP	c-Fos	Rb	p21	DLC1	c-Jun	p8
Normal	10/10	10/10	9/10	1/10	1/10	1/10	7/10	9/10	2/10	10/10
HBV	5/10	7/10	3/10	8/10	9/10	7/10	2/10	5/10	8/10	8/10
HBV + HCC	15/36	24/36	4/36	29/36	34/36	25/36	11/36	7/36	33/36	30/36
X^2	10.5769	4.4179	23.451	18.351	33.4815	11.828	6.4948	19.07	19.6138	0.0100
P value	0.0050	0.1098	< 0.0001	0.0001	< 0.0001	0.0027	0.0389	< 0.0001	< 0.0001	0.995

Abbreviation: HBV: positive Hepatitis B virus; HCC: hepatocellular carcinoma.

Study approval

Animal use was approved by the Institutional Animal Care and Use Committee at the Huazhong University of Science and Technology, China. The Huazhong University of Science and Technology Ethics committee had approved this research involving human participants, and written informed consent was obtained from all enrolled patients in accordance with approval granted by the Huazhong University of Science and Technology, China.

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Conflicts of interest statement

All authors declare that there is no conflict of Interest related to this manuscript. The contents of this manuscript have been approved by all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.020.

References

[1] M.W. Yu, F.C. Hsu, I.S. Sheen, C.M. Chu, D.Y. Lin, C.J. Chen, et al., Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic hepatitis B virus carriers, Am. J. Epidemiol. 145 (1997) 1039–1047.
[2] M. Pinter, M. Trauner, M. Peck-Radosavljevic, W. Sieghart, Cancer and liver cirrhosis: implications on prognosis and management, ESMO Open 1 (2016) e000042.
[3] H. Kojima, K.D. Kaita, Z. Xu, J.H. Ou, Y. Gong, M. Zhang, et al., The absence of up-regulation of telomerase activity during regeneration after partial hepatectomy in hepatitis B virus X gene transgenic mice, J. Hepatol. 39 (2003) 262–268.
[4] M. Blachier, H. Leleu, M. Peck-Radosavljevic, D.C. Valla, F. Roudot-Thoraval, The burden of liver disease in Europe: a review of available epidemiological data, J. Hepatol. 58 (2013) 593–608.
[5] G. Fattovich, F. Bortolotti, F. Donato, Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors, J. Hepatol. 48 (2008) 335–352.
[6] G. Fattovich, L. Brollo, G. Giustina, F. Noventa, P. Pontisso, A. Alberti, et al., Natural history and prognostic factors for chronic hepatitis type B, Gut 32 (1991) 294–298.
[7] F.E. de Jongh, H.L. Janssen, R.A. de Man, W.C. Hop, S.W. Schalm, M. van Blankenstein, Survival and prognostic indicators in hepatitis B surface antigen-positive cirrhosis of the liver, Gastroenterology 103 (1992) 1630–1635.
[8] R. Guan, H.F. Lui, Treatment of hepatitis B in decompensated liver cirrhosis, Int. J. Hepatol. 2011 (2011) 918017.
[9] A. Miyajima, M. Tanaka, T. Itoh, Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming, Cell Stem Cell 14 (2014) 561–574.
[10] V. Lukacs-Kornek, F. Lammert, The progenitor cell dilemma: cellular and functional heterogeneity in assistance or escalation of liver injury, J. Hepatol. 66 (2017) 619–630.
[11] M.H. Tai, C.C. Chang, M. Kiupel, J.D. Webster, L.K. Olson, J.E. Trosko, Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis, Carcinogenesis 26 (2005) 495–502.
[12] K.N. Lowes, B.A. Brennan, G.C. Yeoh, J.K. Olynyk, Oval cell numbers in human chronic liver diseases are directly related to disease severity, Am. J. Pathol. 154 (1999) 537–541.
[13] L. Libbrecht, V. Desmet, B. Van Damme, T. Roskams, The immunohistochemical phenotype of dysplastic foci in human liver: correlation with putative progenitor cells, J. Hepatol. 33 (2000) 76–84.
[14] C. Selden, S.A. Chalmers, C. Jones, R. Standish, A. Quaglia, N. Rolando, et al., Epithelial colonies cultured from human explanted liver in subacute hepatic failure exhibit hepatocyte, biliary epithelial, and stem cell phenotypic markers, Stem Cell. 21 (2003) 624–631.
[15] M.R. Alison, S. Islam, S. Lim, Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly, J. Pathol. 217 (2009) 282–298.
[16] R. Sun, B. Gao, Negative regulation of liver regeneration by innate immunity (natural killer cells/interferon-gamma), Gastroenterology 127 (2004) 1525–1539.
[17] H. Ito, K. Ando, T. Nakayama, M. Taniguchi, T. Ezaki, K. Saito, et al., Role of Valpha 14 NKT cells in the development of impaired liver regeneration in vivo, Hepatology 38 (2003) 1116–1124.
[18] M.M. Richardson, J.R. Jonsson, E.E. Powell, E.M. Brunt, B.A. Neuschwander-Tetri, P.S. Bhathal, et al., Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction, Gastroenterology 133 (2007) 80–90.
[19] K. Kuramitsu, D.Y. Sverdlov, S.B. Liu, E. Csizmadia, L. Burkly, D. Schuppan, et al., Failure of fibrotic liver regeneration in mice is linked to a severe fibrogenic response driven by hepatic progenitor cell activation, Am. J. Pathol. 183 (2013) 182–194.
[20] M.J. Williams, A.D. Clouston, S.J. Forbes, Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion, Gastroenterology 146 (2014) 349–356.
[21] P. Sancho-Bru, J. Altamirano, D. Rodrigo-Torres, M. Coll, C. Millan, J. Jose Lozano, et al., Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis, Hepatology 55 (2012) 1931–1941.
[22] A. Durnez, C. Verslype, F. Nevens, J. Fevery, R. Aerts, J. Pirenne, et al., The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin, Histopathology 49 (2006) 138–151.
[23] J.S. Lee, J. Heo, L. Libbrecht, I.S. Chu, P. Kaposi-Novak, D.F. Calvisi, et al., A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells, Nat. Med. 12 (2006) 410–416.
[24] M.L. Dumble, E.J. Croager, G.C. Yeoh, E.A. Quail, Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma, Carcinogenesis 23 (2002) 435–445.
[25] R.S. Cheung, J.T. Brooling, M.M. Johnson, K.J. Riehle, J.S. Campbell, N. Fausto, Interactions between MYC and transforming growth factor alpha alter the growth and tumorigenicity of liver progenitor cells, Carcinogenesis 28 (2007) 2624–2631.
[26] V.M. de Lima, C.P. Oliveira, V.A. Alves, M.C. Chammas, E.P. Oliveira, J.T. Stefano, et al., A rodent model of NASH with cirrhosis, oval cell proliferation and hepatocellular carcinoma, J. Hepatol. 49 (2008) 1055–1061.
[27] I. Guest, Z. Ilic, S. Sell, Age dependence of oval cell responses and bile duct carcinomas in male fischer 344 rats fed a cyclic choline-deficient, ethionine-supplemented diet, Hepatology 52 (2010) 1750–1757.
[28] F. Zhang, X.P. Chen, W. Zhang, H.H. Dong, S. Xiang, W.G. Zhang, et al., Combined hepatocellular cholangiocarcinoma originating from hepatic progenitor cells: immunohistochemical and double-fluorescence immunostaining evidence, Histopathology 52 (2008) 224–232.
[29] H.A. Dunsford, S. Sell, F.V. Chisari, Hepatocarcinogenesis due to chronic liver cell injury in hepatitis B virus transgenic mice, Cancer Res. 50 (1990) 3400–3407.
[30] S. Sell, J.M. Hunt, H.A. Dunsford, F.V. Chisari, Synergy between hepatitis B virus expression and chemical hepatocarcinogens in transgenic mice, Cancer Res. 51 (1991) 1278–1285.
[31] B. Na, Z. Huang, Q. Wang, Z. Qi, Y. Tian, C.C. Lu, et al., Transgenic expression of entire hepatitis B virus in mice induces hepatocarcinogenesis independent of chronic liver injury, PloS One 6 (2011) e26240.
[32] D. Li, D.Z. Xu, B.C. Choi, K. Men, J.X. Zhang, X.Y. Lei, et al., Preliminary study on the efficacy and safety of lamivudine and interferon alpha therapy in decreasing serum HBV DNA level in HBV positive transgenic mice during pregnancy, J. Med. Virol. 76 (2005) 203–207.
[33] Z. Zheng, J. Li, J. Sun, T. Song, C. Wei, Y. Zhang, et al., Inhibition of HBV replication by theophylline, Antivir. Res. 89 (2011) 149–155.
[34] C.M. Kim, K. Koike, I. Saito, T. Miyamura, G. Jay, HBx gene of hepatitis B virus induces liver cancer in transgenic mice, Nature 351 (1991) 317–320.
[35] T. Roskams, Liver stem cells and their implication in hepatocellular and cholangiocarcinoma, Oncogene 25 (2006) 3818–3822.
[36] C. Brechot, D. Kremsdorf, P. Soussan, P. Pineau, A. Dejean, P. Paterlini-Brechot, et al., Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC): molecular mechanisms and novel paradigms, Pathol Biol (Paris) 58 (2010) 278–287.
[37] T.G. Bird, S. Lorenzini, S.J. Forbes, Activation of stem cells in hepatic diseases, Cell Tissue Res. 331 (2008) 283–300.
[38] J.S. Lee, I.S. Chu, A. Mikaelyan, D.F. Calvisi, J. Heo, J.K. Reddy, et al., Application of comparative functional genomics to identify best-fit mouse models to study human cancer, Nat. Genet. 36 (2004) 1306–1311.
[39] A. Sweet-Cordero, S. Mukherjee, A. Subramanian, H. You, J.J. Roix, C. Ladd-Acosta, et al., An oncogenic KRAS2 expression signature identified by cross-species gene-expression analysis, Nat. Genet. 37 (2005) 48–55.
[40] C. Coulouarn, V.M. Factor, S.S. Thorgeirsson, Transforming growth factor-beta gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer, Hepatology 47 (2008) 2059–2067.
[41] J.B. Andersen, R. Loi, A. Perra, V.M. Factor, G.M. Ledda-Columbano, A. Columbano, et al., Progenitor-derived hepatocellular carcinoma model in the rat, Hepatology 51 (2010) 1401–1409.