SIX1/EYA1 are novel liver damage biomarkers in chronic hepatitis B and other liver diseases

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Abstract

Background: The aim of this study was to investigate the clinicopathological significance of SIX1/ EYA1 in chronic hepatitis B (CHB) and other liver diseases.

Materials and methods: Both SIX1 and EYA1 levels were detected in human serum and liver tissues by enzyme linked immunosorbent assay (ELISA) and immunofluorescent staining, respectively.

Results: Serum SIX1 and EYA1 levels were 7.24±0.11 ng/ml and 25.21±0.51 ng/ml, respectively, in 313 CHB patients, and these values were significantly higher than those in 33 healthy controls (2.84±0.15ng/ml and 13.11±1.01ng/ml, respectively; $P < 0.05$). Serum SIX1 and EYA1 were also significantly increased in patients with many other liver diseases including liver fibrosis, hepatocellular carcinoma, fatty liver disease, alcoholic liver disease, fulminant hepatic failure, autoimmune liver disease, and hepatitis C relative to healthy controls ($P < 0.05$). Dynamic observation of these proteins over time in 35 selected CHB patients revealed that SIX1 and EYA1 serum levels increased over an interval. Immunofluorescent staining revealed that both SIX1 and EYA1 were only expressed in hepatic stellate cells (HSCs), and their increased expression was evident in CHB liver tissue.

Conclusion: Both SIX1 and EYA1 are novel biomarkers of liver damage in CHB and other liver diseases, with potential clinical utility.

Background

SIX1 is a member of the Sine oculis homeobox transcription factor family (SIX1–6) in humans[1, 2]. SIX1 interacts with the transcriptional coactivator and phosphatase EYA1 to form a transcriptional complex that drives the expression of particular genes, thereby controlling cell proliferation and differentiation in numerous organs including the brain, auditory system, lung, muscle, kidney, and craniofacial structures, among others[3, 4].Aberrant SIX1 over-expression in adult tissue is associated with the initiation and progression of many types of cancer, including breast, ovarian, cervical, and liver cancer[5, 6]. Over-expression of SIX1 in hepatocellular carcinoma is significantly correlated with the proliferation and metastasis of tumor cells, resulting a reduced 5-year survival rate in those patients whose tumor cells overexpress this gene [7, 8, 9]. CHB is a viral disease that is particularly common in developing countries, and which can cause liver fibrosis or hepatocarcinoma development in affected patients [10, 11]. In the present study, we collected serum and liver tissue samples from patients with CHB and other liver diseases in order to investigate the clinical significance and physiopathological role of SIX1 and EYA1(Eyes absent 1) in these diseases.

Methods

Patients

313 CHB patients, 153 patients with other liver diseases, and 33 healthy controls were recruited from among out- or in-patients in the Department of Infectious Diseases and Health Management Center from December 8, 2017 to September 13, 2018, and were enrolled in this study. CHB and other liver diseases were diagnosed according to AASLD 2018 hepatitis B guidance and other pertinent diagnostic criteria.

Collection of serum samples and clinical biochemical tests

Serum samples were allowed to clot for two hours at room temperature (RT), then underwent centrifugation at 1000×g for 20 minutes, and supernatants were stored at -80℃ for later analysis. Clinical serological biochemical tests were carried out in the clinical laboratory of the Department of Infectious Diseases, Southwest Hospital.

Dynamic observation of SIX1 and EYA1 serum levels in selected CHB patients before and after comprehensive treatment

Serum samples collected from CHB patients whose alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were over 100 IU/L before antivirus regimens or non-specific therapy and below 100 IU/L after treatment respectively and were tested for SIX1 and EYA1 levels by ELISA. As such, these samples were different from previous samples even from the same patient.

Assessment of SIX1 and EYA1 serological levels by ELISA

Both SIX1 and EYA1 ELISA kits were purchased from Shanghai JiangLai Industrial Limited by Share Ltd., and were used according to provided instructions. Briefly, 50μl of standard or sample was added to appropriate wells, and then100μl of enzyme conjugate was added to wells for 60 minutes at37°C. Plates were then washed 4 times, and 50μl each of substrate A and B were added to each well for 15 minutes at 37°C protected from light. Next, 50μl stop solution was added to each well, and the optical density(O.D) of each well was measured at450 nm using a microplate reader (Multiskan Spectrum 51118650, Thermo Scientific, Waltham, MA, USA) within 15minutes.

Liver tissues

Liver biopsy samples were obtained from a CHB inpatient. Human healthy liver tissues were provided by Xi'an Alenabio. Inc and were used as a positive control. B6 mouse liver was used as negative control.

Immunofluorescent staining for SIX1, EYA1, human serum albumin (HSA), and glial fibrillary acidic protein (GFAP) in frozen liver sections

Frozen sections were fixed with 100% ethanol for 15 minutes and permeabilized using 0.05% Tween20 twice, 2 minutes per treatment. Blocking was performed using 3% bovine serum albumin for 30 minutes at RT. After washing with PBS, sections were incubated with rabbit anti-human SIX1 primary antibody (1:100 dilution) (HPA001893, Sigma, St. Louis, MO USA), rabbit anti-human EYA1 primary antibody (1:100 dilution) (ab85009, abcam, Cambridge, MA, USA) and mouse anti-human HSA primary antibody (1:1000

dilution) (MAB1455, R&D Systems, Minneapolis MN, USA) or mouse anti-human GFAP primary antibody (1:100 dilution) (MA5-12023,ThermoFisher, Waltham, MA, USA) at 4°C overnight. Sections were then incubated with Alexa Fluor®488 donkey anti-mouse IgG and Alexa Fluor®568 donkey anti-rabbit IgG (1:1000 dilution) (A21202, A10042, ThermoFisher, Waltham, MA, USA) for one hour at RT. After washing with PBS, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Finally, coverslips were mounted with anti-fade mounting medium, and immunofluorescent signals were visualized and recorded using an Olympus DP72 microscope and the cellSens Standard 1.5 software.

Protein-protein interaction (PPI) network analysis

A PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes (https://string.embl.de/) database with a medium confidence of 0.40. Combined scores were calculated based on neighborhood, experiment, gene fusion, co-occurrence, text mining, co-expression, and database annotations.

Statistical analysis

Statistical analyses were carried out using the SPSS v17.0 statistical package (SPSS Inc., Chicago, IL, USA), with comparisons being made via student's *t*-tests. $P<0.05$ was considered to be statistically significant.

Results

Increased serum SIX1 and EYA1 levels in patients with CHB and other liver diseases

An ELISA assessment of serum SIX1 and EYA1 levels revealed that levels in 313 CHB patients (Table1) were significantly higher than those in healthy controls ($P < 0.05$). Serum SIX1 levels were also significantly increased in patients with many other liver diseases (Table 2), including liver fibrosis, hepatocellular carcinoma, fatty liver disease, alcoholic liver disease, fulminant hepatic failure, autoimmune liver disease, and hepatitis C ($P < 0.05$). Levels were not increased in those patients with drug-induced liver injury or with inherited liver diseases, including Wilson's disease, hemochromatosis, and hereditary hyperbilirubinemia. Serum EYA1 levels were significantly elevated in patients with all assessed liver diseases relative to healthy controls ($P < 0.05$). Our findings indicated that both SIX1 and EYA1 may play an important role in CHB and other liver diseases.

Dynamic observation of serum SIX1 and EYA1 levels in 35 CHB patients

We next selected 35 CHB patients for the dynamic observation of serum SIX1 and EYA1 over time. We found that the serum SIX1 and EYA1 levels remained significantly elevated in these patients relative to healthy controls, while both ALT and AST levels decreased to below 100 IU/L in these individuals at intervals of 34 to 193 days (Table3). Serum EYA1 levels in patients in the convalescent phase increased

significantly relative to the post-treatment recovery phase (P<0.05). Together these results suggest that SIX1 and EYA1 are involved in liver pathological processes and may enhance liver repair.

HSC-specific elevated expression of SIX1 and EYA1 in CHB liver tissues

GFAP is an intermediate filament protein that is expressed by numerous cell types including astrocytes in the nervous system and hepatic stellate cells (HSCs) in the liver. In this study, we found that both SIX1 and EYA1 staining was detectable in GFAP-positive HSCs but not in HSA-positive hepatocytes based on immunofluorescent staining (Figure 1). The expression of SIX1 and EYA1 in the liver of CHB patients was elevated relative to normal liver controls, in which only 1-4 positive cells were detectable per microscopy field. These findings revealed that both SIX1 and EYA1 were only expressed in HSCs, which play an important role in liver repair and regeneration. Elevated SIX1 and EYA1 expression in CHB liver tissue may be involved in hepatitis B virus-induced pathological progression or disease outcomes.

Protein-protein interaction network analysis

To explore potential molecular biological connections at the protein level, we utilized the STRING database to generate a PPI network. As shown in Figure 2A, there were significant associations between SIX1 and the ten following related genes:EYA1 (combined score=0.983), EYA2 (combined score=0.963), DACH1 (combined score=0.921), EYA4 (combined score=0.906), EYA3 (combined score=0.904), MYOD1 (combined score=0.837), SALL1 (combined score=0.801), PAX6 (combined score=0.770), BMP4 (combined score=0.769), and MYOG (combined score=0.789). For EYA1, ten potential related genes were identified based upon a PPI network (Figure 2B): DACH1 (combined score=0.966), H2AFX (combined score=0.947), MDC1 (combined score=0.931), HIST2H2BE (combined score=0.914), EYA4 (combined score=0.907), EYA3 (combined score=0.904), ATM (combined score=0.914), KAT5 (combined score=0.905), SIX1 (combined score=0.983), and SIX2 (combined score=0.970).

Discussion

Chronic hepatitis B is a form of chronic hepatophagocytic viral infection in humans which induces deleterious protracted immune responses, ultimately leading to progressive liver damage, fibrosis, or cirrhosis. CHB is also known to be a significant risk factor for the eventual development of hepatocellular carcinoma [12, 13]. The accurate estimation of CHB staging and severity is extremely important in clinical settings in order to allow for the correct selection of appropriate therapeutic interventions. Currently, such estimations are made largely based upon serial serological biochemical tests that are intended to reflect the liver injury and repair responses in patients, but these tests are limited in their ability to fully reflect the entirety of liver function in patients [14, 15]. SIX1 is a DNA binding protein that interacts with its coactivator, EYA1, to form a transcriptional complex regulating the cell proliferation and differentiation [1, 2]. SIX1 is also involved in various tumors including those associated with liver cancer, and its expression is associated with increased mortality [16, 17]. At present it is unclear as to whether SIX1 and EYA1 are involved in CHB and other liver diseases. In this study we found, for the first time,that increased serum SIX1 and EYA1 were detectable in patients with CHB and other diseases relative to healthy controls. This

novel finding strongly suggests that both SIX1 and EYA1 may be involved in the pathological development and progression of CHB and other liver diseases. HSCs are well documented to be involved in liver fibrosis, and once activated these cells serve as the primary cellular source of matrix protein-secreting myofibroblasts [18, 19]. They are also known to be involved in liver repair and regeneration [20, 21]. HSC-specific expression of SIX1 and EYA1 in this study indicated that both of these proteins can participate in the liver repair and regeneration in the context of chronic hepatitis B virus infection and other liver diseases. Dynamic observation of serological SIX1 and EYA1 in CHB patients also allowed us to confirm this finding. Up-regulation of SIX1/EYA1 did not correlate with disease status, possibly due to chronic liver damage and repair in the liver of CHB patients. However, further study will be needed to confirm these findings, as the results in the present study remain relatively superficial.

Conclusions

Taken together, Our study found elevated serum SIX1 and EYA1 levels in patients with CHB and other liver diseases. Both SIX1 and EYA1 expressions were HSC-specific and significantly increased in the liver tissue of CHB samples. These results indicate that both SIX1 and EYA1 are novel biomarkers which may be useful for the clinical diagnosis of CHB and other liver diseases, or for determining patient prognosis.

Abbreviations

CHB: chronic hepatitis B; ELISA: enzyme linked immunosorbent assay; HSC: hepatic stellate cell; SIX1: Sine oculis homeobox homolog 1; EYA1: Eyes absent 1; RT: room temperature; ALT: alanine aminotransferase; AST: Aspertate Aminotransferase; ALP: alkaline phosphatase; GGT: Glutamyl transpeptidase; TBIL: total bilirubin; DBIL: direct bilirubin; IB: indirect bilirubin; TP: total protein; HSA: human serum albumin; G: globulin; A/G: ALB/globulin ratio; GFAP: glial fibrillary acidic protein; DAPI: 4’,6-diamidino-2-phenylindole; PPI: Protein-protein interaction;

Declarations

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Author's contributions

JD and PX conceived and designed the study. BX,CZ,YT, ZT, HF, JP, XX and LG collected data. BX performed the experiments. GD and QM contributed as clinicians. JD and ZZ analyzed the data. JD drafted the manuscript . All authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Human Ethics Committee and the Research Ethics Committee of Southwest Hospital, Third Military Medical University. Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Table

Table 1 SIX1 and EYA1 serological levels in CHB and healthy control groups

Characteristics		Age (years old) (mean±SD)	No. of cases (n)	SIX1		EYA1	
				Mean ±SE (ng/ml)	*P*-value	Mean ±SE (ng/ml)	*P*-value
CHB group		**38.38±11.48**	**313**	**7.24±0.11**	**<0.05**	**25.21±0.51**	**<0.05**
Age	≤45		222	7.35±0.14	<0.05	25.28±0.61	<0.05
	45		91	6.98±0.18	<0.05	25.03±0.95	<0.05
Gender	Male		206	7.18±0.50	<0.05	24.93±0.62	<0.05
	Female		107	7.37±0.18	<0.05	25.75±0.893	<0.05
Healthy group		**40.97±7.60**	**33**	**2.84±0.15**		**13.11±1.01**	
Age	≤45		20	2.68±0.13		12.79±1.34	
	45		13	3.08±0.31		13.61±1.58	
Gender	Male		14	3.07±0.28		14.60±1.17	
	Female		19	2.67±0.14		12.02±1.51	

Table 2 SIX1 and EYA1 serological levels in other liver diseases

Liver disease	No. of cases (n)	Age (years old) (Mean±SD)	SIX1		EYA1	
			Mean±SE (ng/ml)	*P*-value	Mean±SE (ng/ml)	*P*-value
Liver fibrosis	**64**	**48.13±10.39**	**7.21±0.25**	**<0.05**	**26.16±1.02**	**<0.05**
Hepatitis B	54		7.25±0.29	<0.05	26.53±1.10	<0.05
Hepatitis C	3		6.88±0.50	<0.05	24.64±5.80	<0.05
Alcoholic	5		6.87±0.78	<0.05	23.84±4.62	<0.05
Autoimmune liver disease	2		6.74±1.16	<0.05	24.76±0.66	<0.05
Drug-induced liver injury	**24**	**48.25±12.04**	**2.05±0.15**	**<0.05**	**35.07±1.64**	**<0.05**
Hepatocellular carcinoma	**23**	**51.04±8.13**	**7.67±0.50**	**<0.05**	**25.97±1.64**	**<0.05**
Hepatitis B	19		8.01±0.55	<0.05	26.23±1.97	<0.05
Other	4		6.08±0.94	<0.05	24.72±1.60	<0.05
Fatty liver disease	**11**	**38.18±7.96**	**6.98±0.62**	**<0.05**	**25.55±3.39**	**<0.05**
Alcoholic liver disease	**9**	**46.56±10.11**	**6.06±0.58**	**<0.05**	**25.48±2.85**	**<0.05**
Fulminant hepatic failure	**8**	**47.63±9.84**	**8.48±0.59**	**<0.05**	**24.99±3.45**	**<0.05**
Hepatitis B						
Inherited liver disease	**7**	**40.43±14.35**	**1.19±0.36**		**26.42±3.94**	
Wilson's Disease	2		2.87±0.59		22.13±0.19	
Hereditary hyperbilirubinemia	2		2.19±1.02		17.95±2.38	
Hemochromatosis	3		1.93±0.65		34.92±6.37	
Autoimmune liver disease	**7**	**47.71±6.05**	**6.40±0.53**		**27.57±3.33**	
Hepatitis C	**7**	**37.86±14.06**	**7.84±0.80**		**26.69±3.16**	
Healthy control	**33**	**40.97±7.60**	**2.84±0.15**		**13.11±1.01**	

Table 3 Dynamic observation of SIX1 and EYA1 serological levels in 35 CHB patients

Measurement	Normal range	Before (Mean±SE)	After (Mean±SE)	*P*-value
SIX1 (ng/ml)	N/A	5.15±0.51	4.09±0.39	**>0.05**
EYA1 (ng/ml)	N/A	18.64±1.33	31.75±1.92	**<0.05**
ALT (IU/L)	0 ~ 42	297.15±68.14	40.22±4.77	**<0.05**
AST (IU/L)	0 ~ 42	166.19±27.87	36.53±3.73	**<0.05**
ALP (IU/L)	34 ~ 114	84.69±4.07	73.31±3.64	**<0.05**
GGT (IU/L)	4 ~ 50	84.80±11.24	66.37±14.28	>0.05
TBIL (μmol/L)	6 ~ 21	34.75±9.74	17.01±1.76	>0.05
DBIL (μmol/L)	0 ~ 6	19.32±7.34	4.44±0.53	>0.05
IB (μmol/L)	3 ~ 16	15.43±2.55	12.57±1.30	>0.05
TP (g/L)	65 ~ 85	75.88±1.26	76.92±0.72	>0.05
HSA (g/L)	38 ~ 51	43.76±0.92	45.58±0.50	>0.05
G (g/L)	20 ~ 40	32.12±0.98	31.34±0.61	>0.05
A/G	1.2 ~ 2.4	1.40±0.05	1.48±0.03	>0.05

Figures

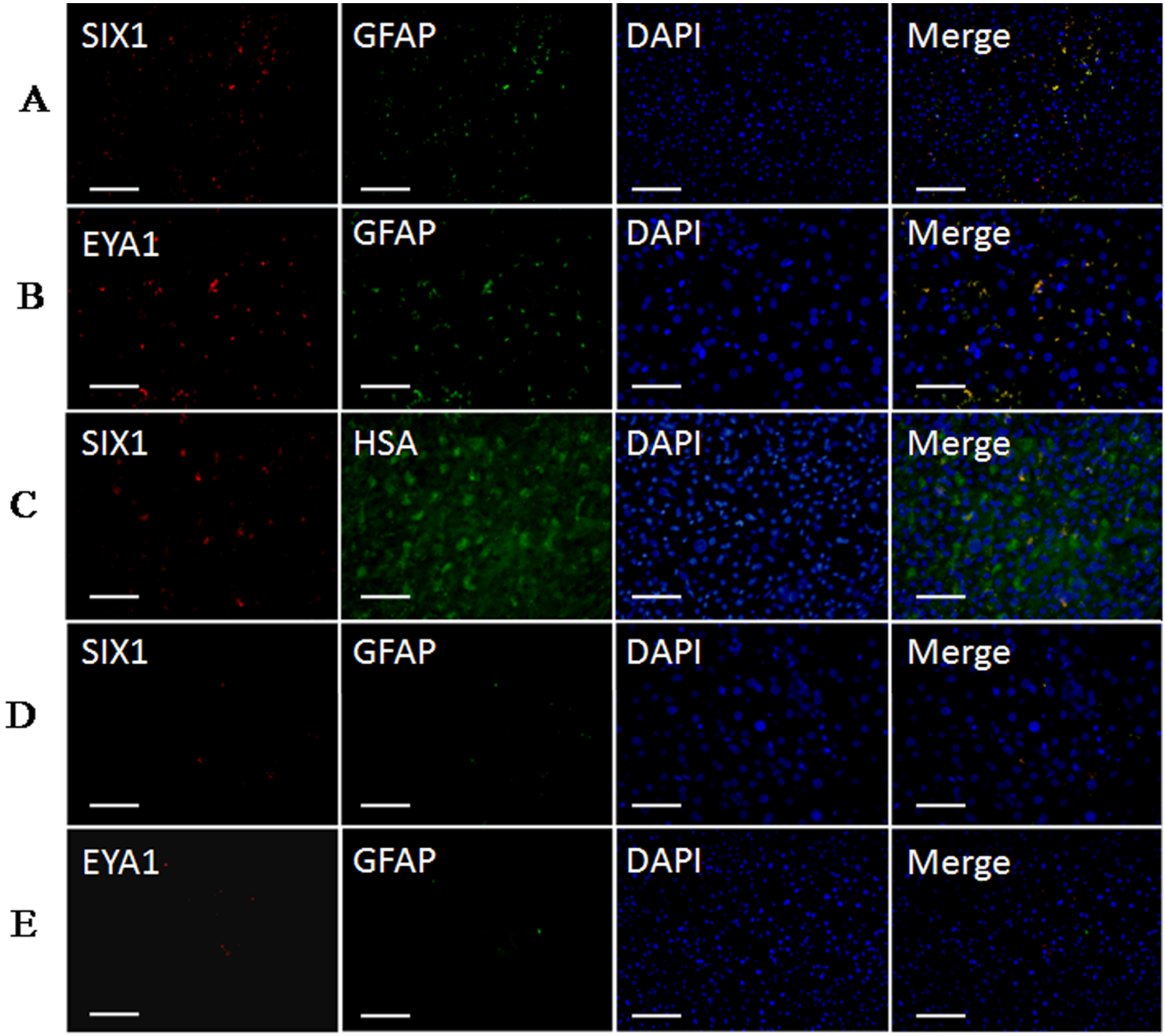

Figure 1

Immunofluorescent staining of SIX1, EYA1, and HSA in liver sections. Panel A, B and C: CHB liver; Panel D and E: healthy control liver. Panel A and D: SIX1 (red) + GFAP (green); Panel B and E: EYA1 (red) + GFAP (green); Panel C: SIX1 (red) + HSA (green). Both SIX1 and EYA1 were expressed in GFAP-positive HSCs (40×magnification). Scale bars: 100μm.

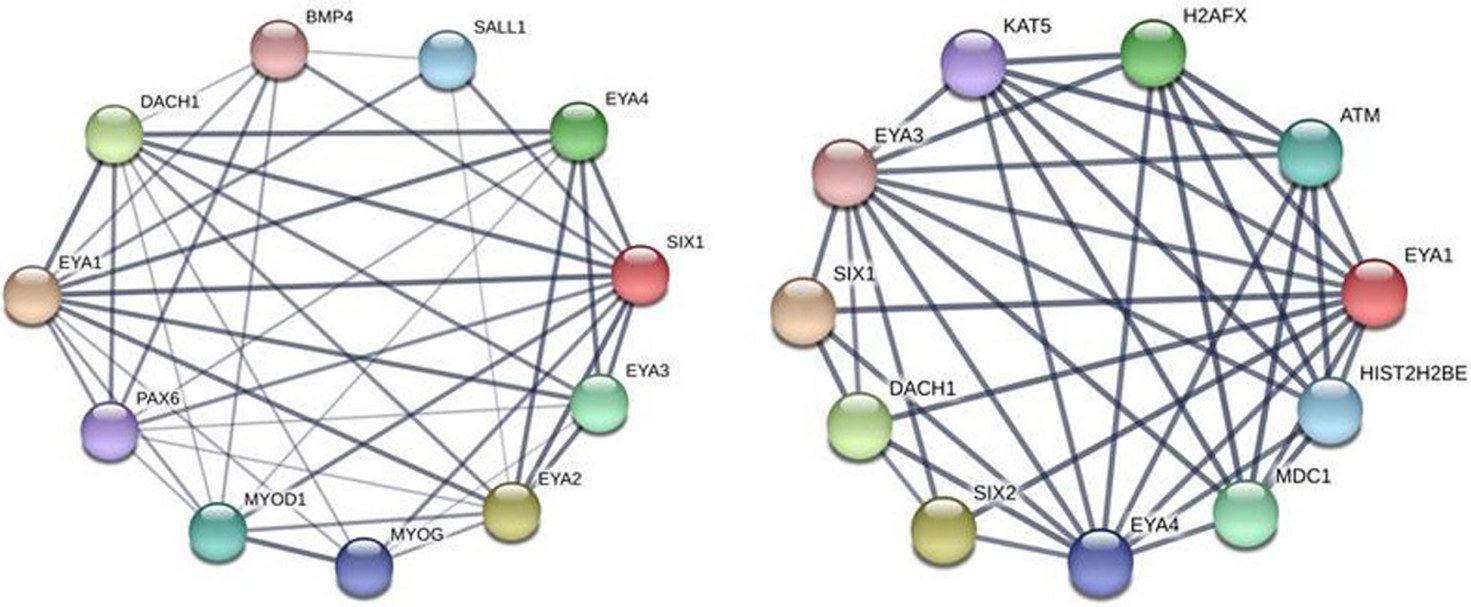

Figure 2

Protein-protein interaction network of SIX1 and EYA1. A. Protein-protein interaction network of SIX1; B. Protein-protein interaction network of EYA1.