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ORIGINAL ARTICLE

Cellular and functional loss of liver endothelial cells correlates with poor hepatocyte regeneration in acute-on-chronic liver failure

Smriti Shubham[1] · Dhananjay Kumar[1] · Sheetalnath Rooge[1] · Jaswinder Sing Maras[1] · Deepanshu Maheshwari[1] · Nidhi Nautiyal[1] · Rekha Kumari[1] · Adil Bhat[1] · Guresh Kumar[1] · Archana Rastogi[3] · Senthil Kumar[4] · Viniyendra Pamecha[4] · Rakhi Maiwall[2] · Chhagan Bihari[3] · Anupam Kumar[1] · Shiv Kumar Sarin[2]

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Abstract

Background and aim Acute hepatic insult triggers regeneration. If acute-on-chronic liver failure (ACLF) patients have a poorer regenerative response than acute liver failure (ALF) patients, and if so, the mechanisms underlying this, are not well understood.

Methods We investigated the status of hepatocyte proliferation, hepatic progenitor cell (HPC) mediated regeneration, non-parenchymal cells (through immunohistochemistery), cytokines and growth factors (cytokine bead array) in liver and peripheral blood of ACLF ($n=29$) and ALF ($n=17$) patients. Liver endothelial cells, mesenchymal cells and Kupffer cells were isolated from explant livers and analysis of regenerative factors was done by qRT-PCR.

Results Unlike ALF, the ACLF livers showed decreased hepatocyte proliferation ($p<0.001$) and profound ductular-reaction with increased CK19 + hepatocytes ($p<0.0001$). However, only decrease in Ki67+ hepatocytes was associated with 28 day mortality in ACLF ($p<0.001$; HR = 0.78; 95% CI 0.69–0.88). In both groups, increase in plasma hepatocyte growth factor (HGF) (OR = 21.87 $p=0.002$;), macrophage colony stimulating factor (MCSF) (OR = 21.73; $p=0.002$) and stromal derived factor (SDF1)(OR = 10.2; $p=0.001$) were associated with hepatocyte proliferation and decreased (> fivefolds) levels were associated with poor hepatocyte regeneration in ACLF patients. ACLF livers showed decrease in endothelial cells ($p<0.01$) and expression of regenerative angiocrine factors C-X-C chemokine receptor type 7 (*CXCR7*), Inhibitor of DNA Binding 1(*IDI*) and *HGF* compared to ALF. In co-culture, while ALF liver mesenchymal stromal cells (LMSCs) induced the expression of *CXCR7, IDI* and *HGF* in human umbilical cord endothelial cells (HUVECs), the ACLF LMSCs were defective and showed decreased production of SDF-1, HGF and MCSF compared to ALF.

Conclusions Decrease in hepatic endothelial cells and their regenerative angiocrine functions indicated by defective CXCR7-ID1 dependent HGF expression underlie the poor hepatocyte proliferation in ACLF compared to ALF patients. A robust hepatocyte self-replication is lacking in the livers of ACLF patients and is associated with poor survival.

Keywords Acute liver failure (ALF) · Liver sinusoidal endothelial cells · Liver regeneration · Hepatocyte proliferation · Hepatic progenitor cells (HPCs) · Hepatocyte growth factor (HGF) · Stromal cell derived factor 1 (SDF1) · Liver progenitors

Introduction

Liver failure is a frequent and often a fatal medical emergency. The incidence of both acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) is rising with the increasing use of alcohol, drugs as acute insults on underlying chronic liver diseases related to obesity and diabetes mellitus [1]. Though liver is an organ with rapid and enormous regenerative capacity, the same is compromised in diseased liver leading to liver failure [2–4].

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Smriti Shubham and Dhananjay Kumar equally contributed.

✉ Anupam Kumar
dr.anupamkumar.ilbs@gmail.com

✉ Shiv Kumar Sarin
sksarin@ilbs.in

Extended author information available on the last page of the article

Restoring the normal regenerative capacity of the liver holds immense promise in the management of acute onset liver failure.

Acute injury to the liver triggers regenerative response and liver can reconstitute its lost mass and hepatic reserve within days to weeks [5, 6]. In ALF, a condition where acute severe hepatic insult occurs in a healthy liver, up to 60% transplant free survival can be achieved with aggressive critical care [7]. However, if acute severe hepatic insult occurs in the presence of chronic liver disease (CLD), a condition known as acute-on-chronic liver failure (ACLF), leads to rapid and progressive liver failure, with a high short-term mortality due to limited functional hepatic reserve [8]. The reason why acute injury in the presence of CLD fails to reconstitute its lost mass and hepatic reserve is not clearly understood.

An optimal regenerative response is critical for the reconstitution of lost hepatic mass after injury. Regeneration in adult liver mainly occurs through the self-replication of existing mature hepatocytes or cholangiocytes or activation and subsequent differentiation of hepatic stem cells when self-replication of hepatic parenchyma is lost or overwhelmed. This complex process of liver regeneration requires a coordinated response of resident non-parenchymal as well as bone marrow cells [9–12]. Recruitment of these cells helps in the mobilization of growth factors, matrix remodelling and a rapid but tightly controlled epithelial and non-parenchymal liver cells proliferation to achieve restitution of liver mass [13]. Earlier our group has shown that hepatocyte proliferation provides the first line of regenerative response in ALF whereas the activation of hepatic progenitor cells (HPCs) is more prominent in ACLF [14]. This finding suggests that the acute insult in healthy liver and in the presence of CLD triggers a differential regenerative response. Underlying cause of this differential regenerative response is not well defined. Potential role of both hepatocytes and ductular cells has been shown in animal model of liver regeneration; however, relative contribution of each of these cells to liver regeneration and recovery of lost hepatic mass is still controversial. Hence, understanding the underlying mechanism of this differential regenerative response and its impact on patient outcome is important for the development of therapeutic strategies to potentiate regeneration in native liver in ACLF patients. The current study was undertaken to investigate the underlying mechanisms of differential hepatic regenerative responses in cases of acute injury in a normal liver as seen in ALF, and in a cirrhotic liver, as seen in patients with ACLF. We studied the liver endothelial cells and the mesenchaymal stromal cells, in particular and assessed their functional regenerative potentials on hepatocytes.

Patients and methods

Patients

The current study included 17 ALF [15] and 29 ACLF [16] patients with histologically proven fibrosis/cirrhosis admitted at the Institute of Liver and biliary Sciences (ILBS), New Delhi, India, from July 2013 to December 2016. The diagnosis of ACLF was based on the APASL criteria [8, 16]. Liver tissue for ALF patients was obtained from explants liver and for ACLF from transjugular liver biopsies/explants liver. Donor liver biopsies ($n=10$) served as controls, which were received for diagnostic evaluation, during donor work-up. Whole blood was obtained from patients before transplant in ALF patients and before transjugular liver biopsy in case of ACLF. No Living donor organs were obtained from executed prisoners or other institutionalized persons.

Immunohistochemistry

Immunohistochemical staining was done as per protocol described earlier [14]. Liver tissue sections were stained using monoclonal antibody for α-smooth muscle actin (α-SMA) (clone HHF35) for liver mesenchymal stromal cells; anti-keratin 19 (clone RCK 108) for ductular cells and Ck19+ hepatocyte (intermediate hepatocyte). AntiKi-67 antibody (clone BGX-297) (for proliferating hepatocyte); CD31 (liver endothelial cells); CD68 (kupffer cells/liver tissue macrophage) (all from Biogenex, CA, USA and CXCR7 (Santa Cruz, USA). Stained slides were observed and analysed by two expert pathologists.

Cytokine bead array

A panel of 45 cytokines, chemokines, and growth factors were measured in plasma of study groups and healthy controls ($n=10$) using a Bio-Plex multiplex cytokine bead assay system (Bio-Rad, CA) as per the manufacturer's protocol. The intra-assay and inter assay coefficient of variation considered for multiplex assays was $<5\%$.

RNA Isolation and qRT-PCR from liver biopsy

Total RNA were isolated from the liver tissue sample or isolated liver endothelial cells, macrophage and mesenchymal stromal cells using the standard TRIZOL method. cDNA was prepared from 1 µg of the total RNA. Total 1 µl of cDNA was used as template and qRT-PCR was performed using SyBr Green master mix and gene specific primers. After the run was complete, the data were exported and analysis was

performed by calculating fold change and plotting graphs. More than two fold differences in relative mRNA expression were considered as significant.

Cell isolation and in-vitro co-culture

Isolation of Liver mesenchymal stromal cell (LMSCs), Liver endothelial cells and liver macrophage from liver tissue of ALF ($n=3$) and ACLF ($n=3$) were done using enzymatic digestion (Collagenase type IV, 0.2 mg/ml) for 30 min at 37 °C with shaking. For LMSC single cell suspension of liver tissue were subjected to ficoll density gradient centrifugation (sample: Ficoll = 4:1). Isolated cells were resuspended and cultured in αMEM with 10% FBS at 37 °C in 5% CO_2. Cells were characterized for stromal cell marker αSMA by immune-florescence staining. Endothelial cells and macrophages were isolated though flow sorting using cell type specific antibody (CD31+ for endothelial cells and CD68 for macrophage) For co-culture experiments, both patient derived LMSCs (0.2×10^6) and HUVECs (0.35×10^6) were plated on transwell inserts and 6-well plates, respectively, and cultured for 48 h at 37 °C in CO_2 incubator. After 48 h HUVECs were trypsinized and collected in RLT buffer for the extraction of total RNA. To quantify the level of different cytokines and growth factors produced by liver stromal cells, equal numbers of cells were plated in 6 well plates at 70% confluence washed with PBS and cultured in serum free media for 24 h. After that supernatant of liver stromal cells were collected and stored at − 80 °C for cytokine quantification.

Statistical analysis

Data were expressed as mean ± SD or median-range. All comparisons were analysed using appropriate parametric and non-parametric statistical tests. Statistics were calculated using SPSS Statistics software version 20.0. Differences were considered as statistically significant if the p value was < 0.05. Univariate and multivariate logistic regression analyses were done to find the factors that could predict regeneration. Cox regression analysis was done to predict the outcome of patient in ACLF at 28 days after admission to the institute. Liver transplant was considered as a competing risk in the Cox-proportional hazard model.

Results

Baseline characteristics of patients

Demographic, clinical, and analytical data of patients admitted with a diagnosis of ALF and ACLF were included and prospectively studied during hospitalization (Table 1). Patients with ACLF had higher incidence of sepsis ($p<0.001$); leukocytosis ($p<0.001$) and higher serum creatinine ($p<0.001$) compared to ALF patients, while the presence of hepatic encephalopathy and ammonia levels ($p<0.001$) were significantly higher in the later group.

Table 1 Clinical and regenerative parameters of patients with ACLF ($n=29$) and ALF ($n=17$)

	ACLF ($n=29$) Mean ± SD	ALF ($n=17$) Mean ± SD	t test p value
Clinical parameters			
Age (years)	44.41 ± 10.92	29.35 ± 12.58	< 0.001
MELD	27.79 ± 9.38	22.35 ± 8.49	0.051
Leucocytes (109/L)	13.76 ± 4.61	9.12 ± 1.58	< 0.001
Hb	10.58 ± 1.66	10.64 ± 2.41	0.922
Platelets (109/L)	*157.66 ± 73.45	143.16 ± 71.16	0.517
INR	1.85 ± 0.39	3.07 ± 1.60	0.007
Blood urea (mg/dl)	44.36 ± 20.08	29.64 ± 10.70	0.002
Serum creatinine (mg/dl)	1.33 ± 0.65	0.47 ± 0.20	< 0.001
Serum Na (mmol/l)	129.65 ± 7.15	137.35 ± 5.41	< 0.001
Serum K (mmol/l)	3.85 ± 0.58	3.39 ± 0.52	0.009
Bilirubin (mg/dl)	25.78 ± 4.31	16.13 ± 3.61	< 0.001
AST (IU/L)	*291.55 ± 112.43	542.00 ± 200.19	< 0.001
ALT (IU/L)	200.10 ± 48.81	311.53 ± 89.85	< 0.001
Albumin (g/dl)	2.20 ± 0.42	2.62 ± 0.66	0.011
Ammonia (mg/L)	146.62 ± 48.46	246.06 ± 74.15	< 0.001
Ascites (%)	89.6	17.6	< 0.001
Baseline sepsis (%)	68.9	17.6	< 0.001
AKI (%)	34.5	23.5	0.415
Regenerative parameters			
ki67%	17 ± 4.22	68.83 ± 11.47	< 0.001
HPC%	47.72 ± 25.42	46.76 ± 22.29	0.898
IH%	46.48 ± 16.31	3.47 ± 2.54	< 0.001
SMA%	52.59 ± 28.25	43.18 ± 19.15	0.186
CD68%	53.89 ± 16.43	62.47 ± 17.07	0.077
CD31%	67.21 ± 16.76	83.82 ± 6.74	< 0.001
CXCR7%	2.62 ± 4.78	39.06 ± 26.77	< 0.001

*Student t test was applied after taking log of values though it is represented as original form

Regenerative responses of ACLF and ALF patients

Regenerative response was measured by analyzing the Ki67+ hepatocytes and CK19+ HPCs and hepatocytes (intermediate hepatocytes, IH) in liver biopsy or explants liver tissue by immunohistochemistry. Number of Ki67+ hepatocytes were significantly ($p<0.001$) reduced in ACLF (Fig. 1a; Table 1) in comparison to ALF, suggesting loss of hepatocyte self-replication (primary mode of hepatic regeneration) in ACLF. Numbers of CK19+ ductular cells/Hepatic progenitor cells (HPCs) were comparable in both

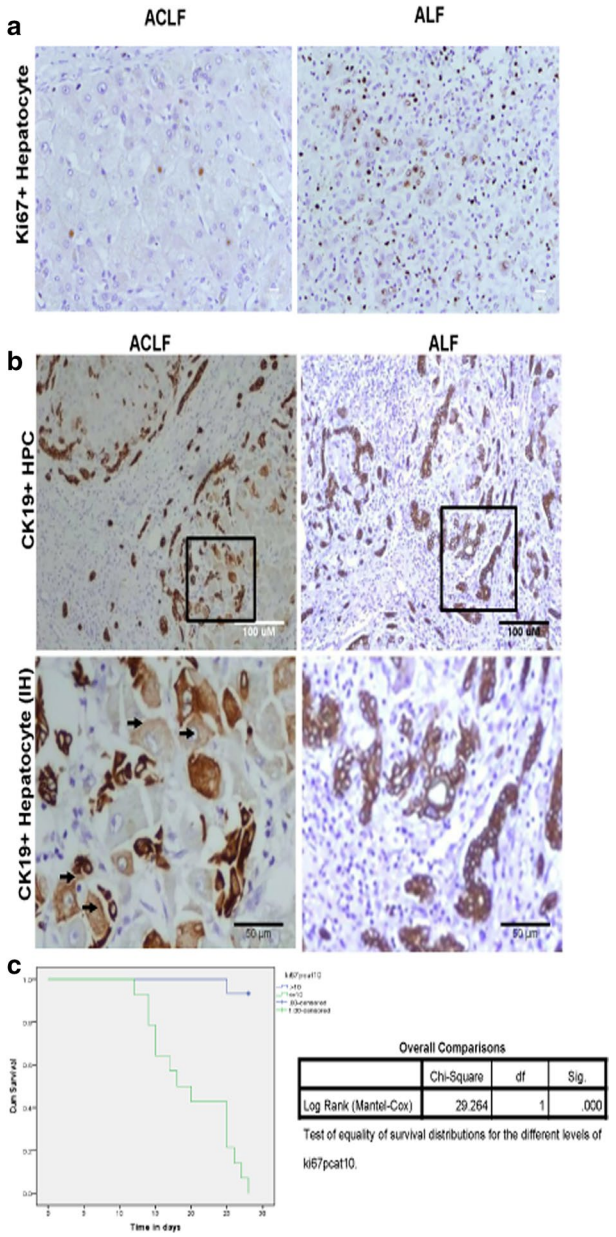


Fig. 1 Immunohistochemical analysis for regeneration status in ACLF and ALF. **a** Representative micrograph showing IHC staining of Ki67+ hepatocytes in ACLF and ALF liver tissue. **b** Representative micrograph showing IHC staining for CK19 for HPCs and IH (intermediated hepatocyte i.e. hepatocyte positive for CK19 black arrow) in ACLF and ALF liver tissue. **c** Kaplan–Meier plot showing survival of patients with ACLF at 28 day with respect to Ki67%

ALF and ACLF (Fig. 1b; Table 1). However, unlike ALF, ACLF liver showed atypical CK19+ cells, not confined to luminal structures but spread in the hepatic parenchyma near portal area (Fig. 1b). ACLF liver also showed increased ($p < 0.001$) number of CK19+ hepatocytes (Table 1) around these CK19+ ductular cells, suggesting HPC mediated liver regeneration in ACLF.

To understand the impact of hepatocyte replication and ductular reaction/HPC mediated regeneration, ACLF patients were further divided into two groups based on their 28-day transplant free survival. At the end of the 28 days follow-up period, 14 (48%) patients were alive (Group-1) and the remaining 15 (52%) had died or had been transplanted (Group-2). As summarized in Table-S1 in univariant cox regression analysis increase in peripheral blood leucocytes count ($p = 0.006$; HR 1.19; 95% CI 1.05–1.34) blood urea ($p = 0.037$; HR 1.026; 95% CI 1.00–1.05), serum creatinine ($p = 0.015$; HR 2.44; 95% CI 1.19–5.02), serum Na($p = 0.025$; HR 1.14; 95% CI 1.02–1.29), ammonia($p = 0.004$; HR 1.02; 95% CI 1.01–1.03) baseline sepsis($p = 0.056$; HR = 4.28; 95% CI 0.11–1.43), acute kidney injury (AKI) ($p = 0.06$; HR 2.78; 95% CI 0.96–19.03) and decrease in Ki67+ hepatocytes ($p < 0.001$; HR 0.78; 95% CI 0.69–0.88) were significantly associated with the poor prognosis of ACLF patients (Table S1). In multivariant step forward cox-regression analysis in clinical parameters, increase in blood urea ($p = 0.004$; HR 1.05; 95% CI 1.01–1.08); ammonia ($p = 0.001$; HR 1.02; 95% CI 1.01–1.03) and leucocytes count ($p = 0.003$; HR 1.3; 95% CI 1.2–1.56) were found to be most significant predictor of poor prognosis of ACLF. However, among the both clinical and regenerative parameter only number of Ki67+ hepatocytes ($p < 0.001$; HR 0.78; 95% CI 0.69–0.88) were most significantly associated with the poor prognosis of ACLF patients (Table S1). This highlights the importance of hepatocyte regeneration in transplant free survival of these patients. At cut-off of 10% Ki67+ hepatocyte it can predict the mortality in ACLF patients with of 93.3% Sensitivity and 100% Specificity (AOROC = 96.6%). ACLF patient with more than 10% Ki67+ hepatocyte showed significantly ($p < 0.001$) increased 28 days transplant free survival than patients with less than 10% Ki67+ hepatocytes (Fig. 1c). HPC mediated regeneration were though significantly high in ACLF in comparison to ALF (Table 1), percentage of both CK19+ HPCs and hepatocytes were comparable in both the groups in ACLF (Table-S1). Having together this data suggests that optimal hepatocyte self-replication mediated liver regeneration is critical for the spontaneous recovery of ACLF patients and HPC-mediated liver regeneration is either not adequate or not contributing in spontaneous recovery of lost hepatic mass in ACLF.

Cytokine levels and relationship with regenerative response

Growth factors and cytokines produced by resident non-parenchymal liver cells and recruited bone marrow cells in response to liver injury have been shown to orchestrate the complex process of liver regeneration [9–13]. To further understand the poor hepatocyte proliferation in ACLF we

analysed the panel of 41 cytokines and growth factors in peripheral blood plasma of same group of patients. Principal component analysis (PCA) documented clear distinction between ACLF and ALF (Fig. 2a) and identified 7 cytokines and growth factors with Variable Important in Projection (VIP) score ≥1.5 and AOROC > 80% (Fig. 2b, Table 2 and Table S2). ACLF patients showed lower plasma levels of HGF, SDF1, MCSF, Interleukin 15 (IL15), alpha-fetoprotein (AFP), and higher plasma levels of Platelet-derived growth factor receptors (PDGF) and Granulocyte–macrophage colony-stimulating factor (GMCSF) in comparison to ALF ($p < 0.01$, Table 2). Among these increase plasma levels of HGF (OR 21.9; $p = 0.002$), SDF1(OR 10.2; $p = 0.001$), MCSF (OR 21.73; $p = 0.002$), IL-15 (OR 2.38; $p = 0.016$), AFP (OR 2.23; $p = 0.003$) and decrease of GMCSF (OR 0.33; $p = 0.011$) were found to be associated with hepatocyte regeneration in univariant logistic regression analysis. In multivariant step forward analysis (model-1) only HGF was significantly ($p = 0.002$; OR 21.87) correlated with hepatocyte regeneration (ROC cut off of > 700 pg/ml; Sensitivity = 100%; Specificity = 99%) (Table 2). In Model 2 when we removed HGF, MCSF alone were most significantly (OR 21.73; $p = 0.002$) correlated with hepatocyte regeneration. Both SDF1 (r 0.741; $p < 0.001$) and MCSF ($r = 0.734$; $p < 0.001$) were significantly correlated with HGF (Table S3). However, on inclusion of interaction between HGF & SDF1 and HGF & MCSF (model3) in multivariant logistic regression analysis only HGF with SDF1 were significantly ($p = 0.001$; $R = 1.28$; 95% CI 1.10–1.48) associated with hepatocyte regeneration. Overall, suggesting that HGF is the most significant regulator of hepatocyte regeneration. While SDF1 interact with HGF to affect hepatocyte regeneration, effect of MCSF on hepatocyte regeneration are independent of HGF. Further q-RT-PCR analysis showed decreased mRNA expression (> fivefolds) of *HGF, SDF1, MCSF* and increased expression (> fivefolds) of *GMCSF* in ACLF liver compared to ALF liver (Fig. 2c), suggesting the decrease production of HGF, SDF1 and MCSF in ACLF liver might be associated with poor hepatocyte regeneration in these patients in comparison to ALF.

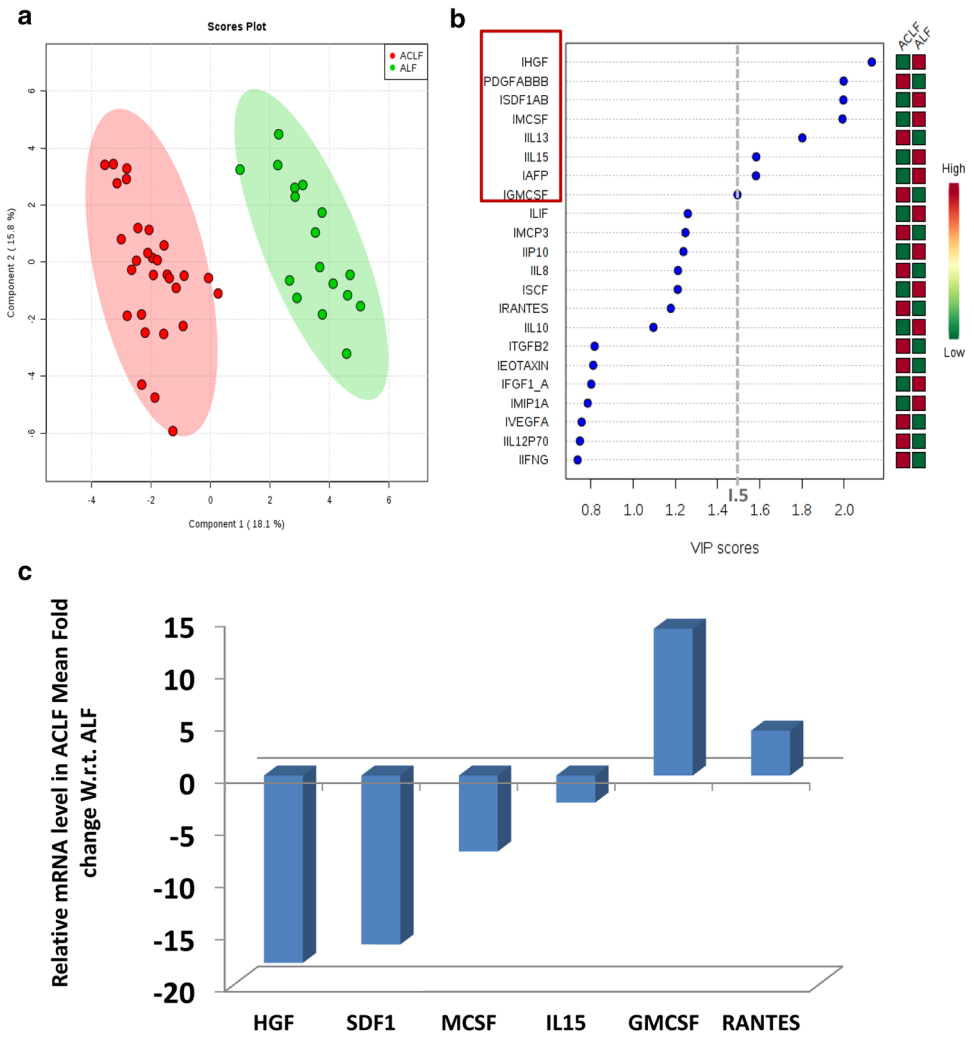


Fig. 2 Cytokines and growth factors in context to regeneration. **a** Principal Component Analysis: plot showing segregation of ACLF and ALF based on cytokines. Confidence region 95% is indicated by an ellipsoid for each group. Each small circle corresponds to one patient. Red circles: patients with ACLF; Green circles: patients with ALF. **b** VIP plot showing variable importance (> 1.5) in projection (VIP) scores for cytokines in the groups. Factors with high in projection (VIP) scores (> 1.5) are regarded as significant. **c** Bar-graph showing relative mRNA level of indicated factors in ACLF liver tissue with respect to ALF (> twofold difference were taken as significant)

Table 2 Peripheral blood plasma level of cytokines, chemokines and growth factors in ALF and ACLF patients having VIP Score ≥ 1.5

S. no.	Cytokines and growth factors	ALF ($n=17$)	ACLF ($n=29$)	*p value	Area (%)	#p value	Cut-off (Pg/ml)	Sensitivity (%)	Specificity (%)
1	HGF	2593.47 (1123.33–19,659.00)	602.82 (111.72–999.32)	< 0.001	100	< 0.001	≥1000	100	96.4
2	MCSF	283.55 (126.48–611.25)	71.03 (13.19–93.19)	< 0.001	100	< 0.001	≥95	100	96.4
3	SDF1	432.88 (189.52–742.31)	122.88 (26.88–184.12)	< 0.001	100	< 0.001	≥175	100	96.4
4	PDGF	427.06 (41.60–1550.02)	1695.55 (1150.00–3925.00)	< 0.001	97.2	< 0.001	≤1250	93.1	94.1
5	GMCSF	7.47 (2.62–13.33)	18.64 (3.22–74.19)	< 0.001	92.1	< 0.001	≤11.5	82.8	82.4
6	IL15	6.47 (1.23–14.05)	2.38 (0.14–6.85)	< 0.001	89.7	< 0.001	≥3.8	76.5	79.3
7	IL13	0.00 (0.00–2.8)	1.00 (0.00–18.30)	0.097	69.8	0.1	≤1.15	58.3	50
8	AFP	7306.00 (1676.68–76,482.00)	3249.54 (1215.58–31,326.00)	< 0.001	91.1	< 0.001	≥3.8	88.2	89.7

*Mann–Whitney Test p value ALF vs ACLF

#p value AOROC

Liver non parenchymal cells and their relationship with regenerative response

In liver, HGF, SDF1 and MCSF are mainly produced by activated HSCs (liver mesenchymal stromal cells(LMSC), endothelial cells and macrophages, so we first check the distribution of these cells in liver tissue by immunohistochemisty using cell type specific (CD31+ endothelial cells; CD68+ liver macrophage; α-SMA + LMSC). Though the number of CD31 + endothelial cells (ALF $p < 0.001$; ACLF $p = 0.003$); α-SMA + LMSCs (ALF $p < 0.001$; ACLF $p < 0.001$) and of CD68 + macrophage (ALF $p < 0.001$; ACLF $p = 0.003$) were significantly increased in both ALF and ACLF in comparison to healthy liver, ACLF liver only showed significant decrease in CD31+ endothelial($p < 0.0001$) in comparison to ALF (Fig. 3a, b; Table 1). No statistically significant difference was observed in α-SMA + LMSCs and CD68+ liver macrophage (Fig. 3a, b and S1; Table 1). To further understand the loss of HGF, SDF1 and MCSF in ACLF we isolated liver endothelial cells, macrophage and mesenchymal stromal cells (LMSCs) (Figure S2abc) from both ACLF and ALF liver and analyzed the expression of these factors by qRT-PCR. In comparison to ALF, ACLF, LMSCs showed significant decrease in expression of *HGF* (> twofold), *SDF1* (> tenfold), *MCSF* (> sixfold). Similarly ACLF liver endothelial cells showed significant decrease in the expression of *HGF* (> tenfold) in comparison to ALF (Fig. 3c). All together these data suggest the compromised production of HGF by liver endothelial cells and SDF-1, MCSF by LMSCs may be associated with poor hepatocyte regeneration in ACLF.

ACLF liver endothelial cells are defective in CXCR7-ID1 dependent HGF expression

Since HGF was most significantly associated with hepatocyte proliferation (Table 3) and ACLF liver endothelial cells showed significant (> tenfold) loss *HGF* expression in comparison to ALF (Fig. 3c). To further understand the compromised HGF production in ACLF we analyzed the expression of upstream target genes (CXCR7, CXCR4 and ID1) responsible for the expression of *HGF* in liver endothelial cells [17]. Indeed our qRT-PCR data showed significant decrease in expression of *CXCR7* (> tenfold), *ID1*(> fivefold) and increase in *CXCR4* in ACLF liver endothelial cells in comparison to ALF (Fig. 4a) which was corroborated by a significant loss of CXCR7+ endothelial cells in ACLF ($p < 0.001$) in comparison to ALF (figure S3) suggesting the compromised CXCR7-ID1-HGF dependent regenerative angiocrine function of ACLF liver endothelial cells in comparison to ALF. Since we also observed functional defect in ACLF liver mesenchymal stromal cells in terms of expression of growth factors associated with hepatocyte proliferation (Fig. 3c), to check whether LMSC modulate the regenerative function of endothelial cells, we co-culture LMSCs isolated from ALF and ACLF liver with HUVECs and analyze the expression of CXCR7, ID1 and HGF by q-RT-PCR. While ALF, LMSCs significantly induce the expression of CXCR7 (> fivefold), ID1 (> 12 fold) and HGF

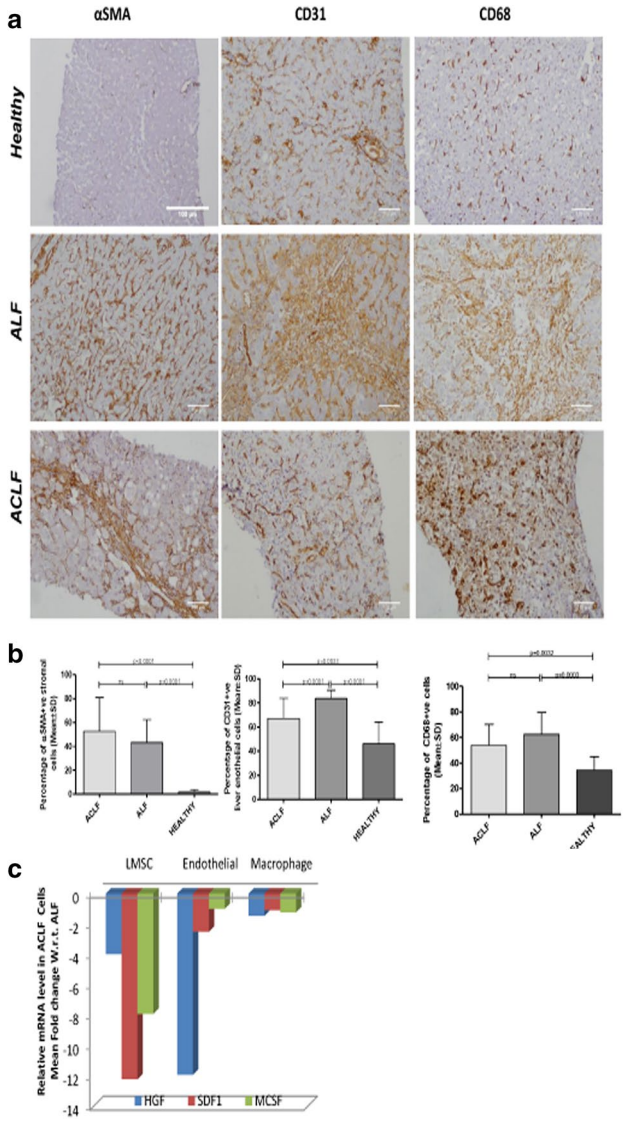


Fig. 3 Immunohistochemical analysis for nan-parenchymal cell status in Healthy, ALF and ACLF liver: **a** Representative micrograph showing IHC staining of αSMA + LMSC; CD31+ endothelial cells and CD68+ liver macrophage (**b**) bar-diagram showing % of αSMA + LMSC; CD31+ endothelial cells and CD68 + liver macrophage Statistical non-parametric student *t* test was used for comparison. **c** Bar-graph showing relative mRNA level of *HGF* (blue), *SDF-1*(Red) and *MCSF* (green) in LMSC, endothelial cells and macrophage of ACLF liver in comparison to ALF (> twofold difference were taken as significant)

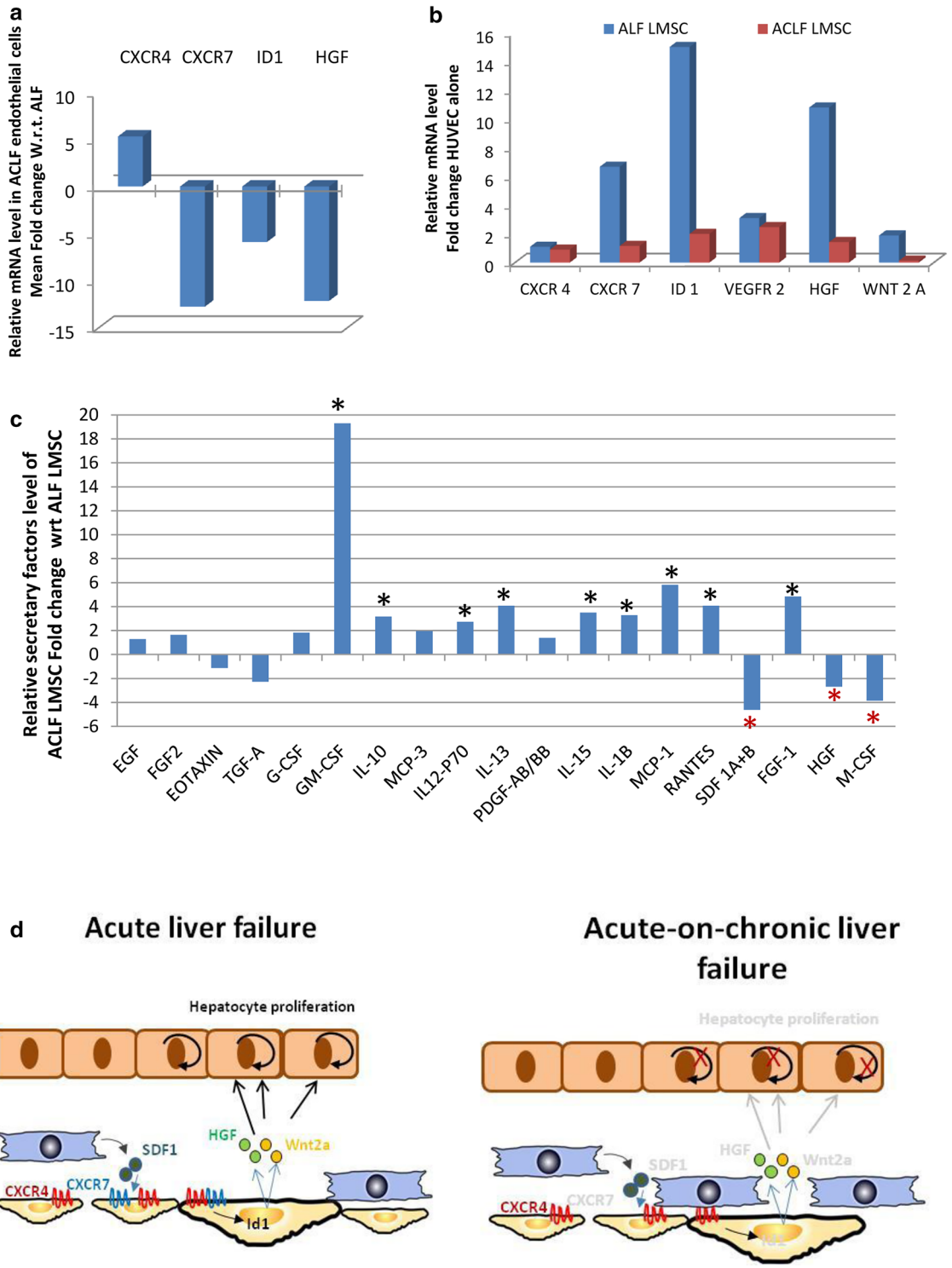
(> tenfold) expression in HUVEC, ACLF, LMSCs found to be grossly defective in the induction of CXCR7-ID1-HGF axis (Fig. 4b). Further analysis of conditioned media from ACLF and ALF liver stromal cells showed > twofolds increase in the levels of GM-CSF, IL10, IL12, IL13, IL1B, MCP1, RANTES and FGF1 and decreased levels of SDF-1, HGF, and M-CSF in ACLF liver stromal cell in comparison to ALF (Fig. 4c). Overall, these data suggest that ACLF liver endothelial cells are defective in CXCR7-ID1 dependent HGF expression. Liver mesenchymal stromal cells induce the expression of *CXCR7, ID1* and *HGF* in endothelial cells but this induction is grossly defective in ACLF.

Table 3 Logistic regression analysis of cytokines/growth factors with hepatocyte regeneration in Alf and Aclf (median Ki67 + hepatocyte = 27%; Regeneration > 27%; no regeneration < 27%)

	Univariant logistic regression		Multi variant logistic regression	
	p value	OR (95% CI)	*p* value	OR (95% CI)
Model-1				
HGF	0.002	21.87 (3.15–150)	0.002	21.87 (3.15–150)
MCSF	0.002	21.73 (3.08–153.6)		
SDF1	0.001	10.2 (2.6–39.9)		
AFP	0.003	2.23 (1.131–3.80)		
IL15	0.016	2.38 (3.15–150)		
GMCSF	0.011	0.33 (0.14–0.78)		
Model-2				
MCSF	0.002	21.73 (3.08–153.6)	0.002	21.73 (3.08–153.6)
SDF1	0.001	10.2 (2.6–39.9)		
AFP	0.003	2.23 (1.131–3.80)		
IL15	0.016	2.38 (3.15–150)		
GMCSF	0.011	0.33 (0.14–0.78)		
Model-3				
HGF	0.002	21.87 (3.15–150)		
MCSF	0.002	21.73 (3.08–153.6)		
SDF1	0.001	10.2 (2.6–39.9)		
AFP	0.003	2.23 (1.131–3.80)		
IL15	0.016	2.38 (3.15–150)		
GMCSF	0.011	0.33 (0.14–0.78)		
HGF*SDF1 HGF*MCSF	–	–	0.001	1.28 (1.1–1.48)

Discussion

Efficient regeneration is critical for the reconstitution of lost hepatic mass and spontaneous recovery of patients after hepatic injury. Acute injury to the liver triggers regenerative response [5, 6], however, in ACLF it leads to rapid and progressive liver failure, with a high short-term mortality



due to limited functional hepatic reserve [8] and inability for rapid and adequate hepatic regeneration.

The results of this novel study show that hepatocyte proliferation mediated liver regeneration is critical for spontaneous recovery of ACLF patients. In comparison to ALF, ACLF liver showed significant loss of hepatocyte proliferation with decrease in liver endothelial cell number and their regenerative angiocrine functions.

Studies from animal model of liver injury show that regeneration in adult liver mainly occurs through the self-replication of existing hepatocytes/cholangiocytes or activation and subsequent differentiation of hepatic stem cells

◂**Fig. 4** Comparison of regenerative angiocrine function of ACLF and ALF liver endothelial **a** Bar-graph showing relative mRNA level of *CXCR4*, *CXCR7*, *ID1* and *HGF* in ACLF liver endothelial cells with respect to ALF(> twofold difference were taken as significant). **b** Bar-graph showing relative mRNA level of *CXCR4*, *CXCR7*, *ID1*, *HGF, VEGFR2, WNT2A in* HUVEC cells co-cultured with ALF (blue) and ACLF (red) LMSC with respect to untreated HUVEC [> twofold difference were taken as significant) (**c**)] Bar-graph showing relative level of cytokines and growth factors in conditioned media from ACLF liver mesenchymal stromal cells (LMSC) in comparison to and ALF liver mesenchymal stromal cells. (> twofold difference was taken as significant). **d** Diagram illustrating the putative mechanism of liver regeneration in ALF and ACLF. In ALF liver tissue Liver mesenchymal stromal cells produce SDF1 and endothelial cells express CXCR7 leading to CXCR7-ID1 dependent HGF production and active hepatocyte proliferation. In ACLF compromised SDF1 production by liver mesenchymal stromal cells leads to the loss of CXCR7-ID1-HGF mediated regenerative angiocrine support and hepatocyte proliferation

when self-replication of hepatic parenchyma is lost or overwhelmed [6, 18, 19]. With poor hepatocyte replication though ACLF liver showed significant increase in ductular reaction (an indicative of HPC mediated regeneration) in comparison to ALF, only numbers of Ki-67+ hepatocytes were associated with the prognosis of the ACLF patients and shown to predict mortality with 93.3% sensitivity and 100% specificity (AOROC = 96.6%). Percentage of both CK19+ HPCs and hepatocytes were comparable in both the groups in ACLF with and without 28 days transplant free survival (Table S1). The magnitude of ductular reaction in human liver disease has been shown to increase with the severity of liver disease [20–23]. Even the series of lineage-tracing data in mice have shown limited contribution of these cells in hepatocyte regeneration [24–30].In contrast to these mature hepatocytes have been also shown to reprogram into ductular cells and cholangiocyte under the influence of high notch or biliary injury [31, 32]. All these findings and our current observation of redundant role of ductular cells in the prognosis of ACLF patients suggest that optimal hepatocyte self-replication mediated liver regeneration is critical for the restitution of lost hepatic mass in ACLF. HPC-mediated liver regeneration is either inadequate or not contributing in spontaneous recovery of lost hepatic mass in ACLF.

The complex process of liver regeneration is orchestrated by various growth factors; cytokines and ECM proteins produced by resident non-parenchymal cells and recruited bone marrow cells that lead to a tightly controlled proliferation of epithelial and non-parenchymal liver cells to achieve restitution of lost liver cell mass [9–13]. Our data showed that plasma level of HGF, SDF1 and MCSF are significantly associated with Ki67+ hepatocyte number in both ACLF and ALF liver. HGF is known direct mitogens of hepatocyte. In comparison to ALF, ACLF liver showed significant reduction in expression of HGF by liver endothelial cells. In animal model of liver injury CXCR7-ID1 dependent production of HGF by liver endothelial cells has been shown to be critical for the hepatocyte proliferation [17, 33]. Indeed our data showed significant reduction in number as well as expression of CXCR7 and *ID1* in ACLF liver endothelial cells in comparison to ALF, highlighting the loss of endothelial regenerative angiocrine support in ACLF. This might be responsible for compromised hepatocyte proliferation in these patients in comparison to ALF.

CXCR7 is an SDF-1 responsive gene and platelet-derived SDF-1 has been shown to induce CXCR7 in the pulmonary endothelium [34]. We observed a significant decrease in plasma SDF-1 level in ACLF in comparison to ALF but the platelet counts in both ALF and ACLF patients were comparable. Liver endothelial and mesenchymal/stromal cells are the major source of SDF-1/CXCL12 in liver [35, 36]. Our data showed that liver stromal cells from ALF patients induced the expression of *CXCR7, ID1* and *HGF* in HUVEC. Unlike ALF, isolated liver stromal cells of ACLF fail to induce the expression of CXCR7, ID1 and HGF in HUVEC and shown significant decrease in the production of SDF-1, HGF and MCSF in comparison to ALF. This suggests the potential role of liver stromal/mesenchymal cells in the induction of regenerative angiocrine function of endothelial cells and defective production of SDF-1 by liver stromal cells might be responsible for loss of pro-regenerative function of endothelial cells in ACLF.

In summary, our data showed that hepatocyte self-replication mediated regeneration is essential for spontaneous recovery and survival in ACLF. We identified loss of liver endothelial cells and their CXCR7-IDI-HGF dependent regenerative angiocrine function as one of the probable mechanism of poor hepatocyte replication in ACLF (Fig. 4d). We also showed that liver mesenchymal stromal cells can induce the regenerative angiocrine function of endothelial cells which are grossly defective in ACLF LMSC. Hence, supplementing the healthy Liver mesenchymal cells in ACLF may improve the native liver regeneration by augmenting the regenerative angiocrine function of liver endothelial cells. Mesenchymal stem cells derived from liver tissue have been shown to improve fibrosis in animal model of chronic liver injury [37]. Bone marrow mesenchymal stem cells also produce HGF, SDF1 and MCSF. Mesenchymal Stem cell therapy has recently shown to improve the outcome of ACLF patients [38].

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Compliance with ethical standards

Conflict of interest Smriti Shubham, Dhananjay Kumar, Sheetalnath Rooge, Jaswinder Sing Maras, Deepanshu Maheshwari, Nidhi Nau-

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Affiliations

Smriti Shubham[1] · Dhananjay Kumar[1] · Sheetalnath Rooge[1] · Jaswinder Sing Maras[1] · Deepanshu Maheshwari[1] · Nidhi Nautiyal[1] · Rekha Kumari[1] · Adil Bhat[1] · Guresh Kumar[1] · Archana Rastogi[3] · Senthil Kumar[4] · Viniyendra Pamecha[4] · Rakhi Maiwall[2] · Chhagan Bihari[3] · Anupam Kumar[1] · Shiv Kumar Sarin[2]

[1] Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences (ILBS), New Delhi 110 070, India

[2] Department of Hepatology, Institute of Liver and Biliary Sciences (ILBS), New Delhi 110 070, India

[3] Department of Pathology, Institute of Liver and Biliary Sciences, New Delhi, India

[4] Department of HPB Surgery, Institute of Liver and Biliary Sciences, New Delhi, India