

Evaluation of Inflammatory Cytokine IL-17 and Immunohistochemical Analysis of Cirrhotic Liver from HCV Patients

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ABSTRACT

A distinctive feature of chronic hepatitis C infection is the presence of persistent liver inflammation characterized by the expression of different inflammatory molecules including Interleukin-17 (IL-17). The progression of chronic liver ailments (fibrosis, cirrhosis) towards hepatocellular carcinoma (HCC) is determined by the distortion produced in the normal structure of liver. Pakistan bears the second highest numbers of hepatitis C virus (HCV) infections after Egypt. Although the role of IL-17A in chronic liver diseases (CLD) had been extensively studied, there is little to no data available on the serum profiling of IL-17A in HCV patients from Pakistan. Thus, we investigated the relationship between serum levels of IL-17A and age in chronic and fibrotic HCV patients by ELISA and evaluated different markers that are involved in the progression of CLDs towards HCC by performing histopathology and immunohistochemistry (IHC) techniques in liver tissues in HCV patients from the southern districts of Khyber Pakhtunkhwa, Pakistan. In addition, the network of IL-17A and its KEGG pathway related gene IDs were constructed using GeneMANIA and STRING online web tools. IL-17A serum levels were significantly higher in fibrotic patients compared to chronic HCV patients and showed a positive correlation with age. The constructed gene network revealed interactions between IL-17A and various other genes, especially chemokines. IHC analysis revealed significant changes in the architecture of liver especially an abnormal expression of alpha (α)-SMA and the activation and transmigration of Kupffer cells towards the septa.

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Authors' Contribution

HA and BNM conceptualized and supervised the experimental procedures. SM, AA and MS performed the experiments. HA and NK interpreted the data. SM prepared initial draft of the paper. BNM corrected the initial draft. HA and NK finalized the draft before submission.

Key words

IL-17A, HCV, Cirrhosis, Immunohistochemistry, Interlinkin-17

INTRODUCTION

The hepatitis C virus (HCV) infections have been estimated at around 80 (64–103) million infections globally, among which genotype (G) G1 is the most common, followed by G3, G2, and G4 (Gower *et al.*, 2014). Once contracted, as many as 50–85% of the patients fail to clear the HCV resulting in chronic liver diseases (CLDs) leading to fibrosis and around 15–30% of the chronically infected patients progress to cirrhosis of liver over the ensuing three decades (Zaltron *et al.*, 2012). There is evidence that chronic HCV infection is associated with an abnormal immune activation and senescence which contributes to

increased risks for ongoing damage in the liver. Particularly, cytokines and chemokines attract leucocytes to the site of injury in the liver, enhancing intrahepatic inflammation (Naggie, 2017; Zeremski *et al.*, 2007). Histologically confirmed chronic hepatitis C (CHC) patients from 10 clinical centers in Australia, France, Italy, Switzerland, and the USA, revealed the presence of fibrosis in 87.6% of total patients, suggesting a role for hepatic inflammation in mediating fibrogenesis (Leandro *et al.*, 2006). Various inflammatory molecules, for instance, transforming growth factor (TGF)- β and tumor necrosis factor (TNF)- α (Nueman *et al.*, 2012), interleukin 6 (IL-6) (Malaguarnera *et al.*, 1997), IL-8 (Polyak *et al.*, 2001), IL-22 (Dambacher *et al.*, 2008), IL-32 (Moschen *et al.*, 2011), caspases (Bantel *et al.*, 2001), levels have been shown to be up-regulated with the severity of the chronic HCV pathogenesis, persistence, inflammation, liver injury, and/or fibrosis. Genetic polymorphisms in certain pro-inflammatory cytokines i.e., IL-1 and IL-1ra have also been linked with the presence of HCC (Wang *et al.*, 2003). In addition, chronic inflammation in HCV infections have been associated with altered hepatocytes signaling (e.g.,

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TGF signaling) from tumor-suppression to fibrogenesis (Matsuzaki *et al.*, 2007).

IL-17 is a family of pro-inflammatory cytokines consisting of IL-17 A to F. IL-17A is mainly produced by T helper 17 (Th17) cells, whereas other members of the IL-17 family have multiple sources (from immune to non-immune cells). In case of an uncontrolled immune response, IL-17 may contribute to the pathology and development of chronic inflammation by promoting neutrophil chemotaxis and angiogenesis (Gu *et al.*, 2013). The serum levels of IL-17 is elevated which correlates with HCV-associated grades of inflammation and severe fibrotic stages in liver and HCC tissues (Elbanan *et al.*, 2020; Wu *et al.*, 2017). Circulating and liver-infiltrating Th17 cells in CHC patients increased in proportions and correlated with the severity of liver inflammation and damage compared to healthy individuals (Chang *et al.*, 2012). Similarly, liver biopsies from chronic HCV patients with different liver fibrosis stages had increased the number of IL-17+ cells. The molecular mechanisms of the IL-23 and Th17 cells role in chronic HCV infected patients showed an increased frequency of IL-17A -producing PBMCs (Meng *et al.*, 2016).

HCC is highly heterogeneous both at the molecular and histological level. Several HCC subtypes characterized by histological features have also been identified. For example, HCC comprises of intrahepatic cholangiocarcinoma (iCCA), and other rare tumors i.e., hepatoblastoma and fibrolamellar carcinoma etc., with overlapping risk factors and pathways of oncogenesis (Calderaro *et al.*, 2019; Sia *et al.*, 2017). This array of phenotypical features is expected to ultimately provide information about cell of origin, tumor behavior and, treatment sensitivity (Roncalli *et al.*, 2010). Better understanding of tumor biology has enabled identification of a multitude of pathological and molecular events that drive hepato-carcinogenesis leading to discovery of numerous potential biomarkers that also help in prediction of prognosis or recurrence (Behne and Copur, 2012). The severity of HCC arises from its difficulty to detect and treat. Approximately 70% of HCC cases are caused by HBV and/or HCV infection. Although DAAs can control the replication of these viruses, HCC occurrence is might still be observed. Other major etiologies in HCC are liver fibrosis or cirrhosis from these chronic viral infections and/or accumulation of multiple genetic aberrations caused by pathologically derived liver damage results in carcinogenesis (Kanda *et al.*, 2019; Black and Mehta, 2018; Umeda *et al.*, 2019). Data emerging from small rodent models of CLD have demonstrated that fibrotic extracellular matrix can be remodeled near-normal hepatic architecture upon liver injury (Ellis and Mann, 2012). The cause diminished regeneration, especially in liver cirrhosis, is still unknown. Epithelial-mesenchymal

transition (EMT) has been found to be associated with liver fibrosis. This possibility also reinforces the idea that there are multiple mechanisms involved in the hepatic fibrogenic process (Lee *et al.*, 2014). Similarly, the localized infiltration and accumulation of neutrophils and monocytes at injury site have been reported. Kupffer cells (KCs) were critical for the recruitment of hepatic stellate cells (HSCs) during tissue repair in liver injury (Abbas *et al.*, 2020). However, previously it has been shown that KCs are associated with hepatocellular apoptosis, inflammation, and fibrosis process in rat model of liver fibrosis. In their study, the double α -SMA- and collagen type I-positive cells predominantly existed in fibrotic septa, and those cells were co-localized clearly with CD68-positive cells (Liu *et al.*, 2010). IL-17 induces liver fibrosis through multiple mechanisms in mice. Increased levels of IL-17A and its receptors in response to liver injury have been shown to apparently promote fibrosis by activating inflammatory and liver resident cells. Furthermore, IL-17A directly induced production of collagen type I in HSCs by activating the STAT3 signaling pathway. Mice devoid of STAT3 signaling in HSCs were less susceptible to fibrosis (Meng *et al.*, 2012).

Pakistan bears the second largest burden of HCV infections after Egypt. The estimates of prevalence in Pakistan during 1989-2016 showed that more than 6% of the general population is infected with HCV, which constitutes 55.9% of liver-related ailments. Only minor differences exist in HCV genotypes diversity among the provinces, and G3 is commonly found across the country (Al-Kanaani *et al.*, 2018). With such a high prevalence of persistent infections, there is a greater risk of severe liver damage from low grade but persistent inflammation to those infected from HCV in Pakistan. Thus, there is need for clarifying the influence of inflammation in these chronically infected patients. Current study was, therefore, conducted to evaluate the levels of inflammatory marker IL-17A in chronic and fibrotic HCV infected Pakistani patients belonging to the Khyber Pakhtunkhwa (KPK) province of Pakistan. In addition, those with advance fibrosis or cirrhosis, their liver samples were evaluated by histology and immunohistochemistry techniques to elucidate the damage pattern in liver tissue as a result of HCV infection.

MATERIALS AND METHODS

Sample collection and processing

The current study was conducted from November 2020 to March 2021 at the Department of Biosciences, COMSATS University Islamabad, Pakistan, in collaboration with the Pathology Laboratory of the Pakistan

Institute of Medical Sciences (PIMS) Islamabad, and the Genomics Lab Rawalpindi, Pakistan. Ethical approval was obtained from the institutional ethics review committee (CUI/Bio/ERB/12-19/29). A total of 80 age-sex matched HCV positive patients (40 chronic and 40 fibrotic) who had ALT, ALP, total bilirubin, viral load performed were recruited for the current study, whereas informed consent was obtained from all patients. All patients belonged to the southern region of the KPK. Blood samples taken from the patients were immediately transported on ice to the Genomics Lab Rawalpindi for further analysis. Liver biopsies were processed and evaluated at the Pathology Lab, PIMS Islamabad. Paraffin-embedded formalin-fixed tissues were stained with hematoxylin and eosin (H and E) and Masson trichrome stains for hepatic fibrosis and analyzed at 20x using light microscopy. Besides, 15 patients found cirrhotic were further analyzed by immunohistochemistry. Serum IL-17A was measured by using ELISA kit (Elabscience specific for Human IL-17A) according to the manufacturer guidelines. The correlation between serum IL-17A versus age was analyzed by Pearson correlation regression analysis, where a probability value less than 0.05 was considered statistically significant. Furthermore, an input list of the gene IDs from KEGG pathway was provided to GeneMANIA and STRING database separately and the generated networks were then imported to Cytoscape for visualization purpose. Circular layout was used for STRING network and preferred layout was applied to GeneMANIA network.

Immunostaining procedure

For immunohistological analysis, 5µm thick liver sections of FFPE tissue of all studied cases were examined. Paraffin embedded liver tissues were thoroughly washed in xylene to deparaffinize then followed for rehydration and dehydration using graded alcohol. Finally the tissue sections were put in PBS for few min and were applied a number of rabbit polyclonal antibodies for evaluation of Arginase-1, CD68, alpha SMA, Ki-67 (Santa Cruz, Europe). Primary antibodies after incubation was rinsed in PBS and antigen retrieval was executed with citrate buffer for 15 min at 42°C. The activities of endogenous peroxidase were blocked with hydrogen peroxide for 10 min and briefly washed in buffer. After incubation the sectioned was exposed for mouse monoclonal secondary antibodies conjugated with TxR and FITC and some antibodies detected with the avidin-biotin kit. It is followed by incubation with antibodies (arginase-1) for 50min at room temperature. The antibodies were detected with DAB reaction and counterstained the sections with hematoxylin for 10 min. Normal liver tissues was used as positive control, while negative control was done using the

same tissue (normal liver), omitting the primary antibody.

RESULTS

Biochemical analysis of HCV individuals

In this comparative analysis, a total of 80 HCV positive individuals; 40 chronic and 40 fibrotic were included. The mean age of the participants of chronic group was 41.87 ± 5.59 and fibrotic was 42.27 ± 5.40 years. Other characteristics i.e., ALT, ALP, viral load, and total bilirubin levels of the chronic and fibrotic patients are given in Table I. For the fibrotic group, Masson trichrome staining revealed appreciable presence of fibrosis in liver biopsies and extensive collagen deposition with pseudo-lobular formation (Fig. 3D). It also showed congestion of surrounding hepatocytes and huge abnormalities in the liver architecture of fibrotic patients. In the region of bridging of fibrosis, there was presence of elongated nuclei cells which represents the infiltration of inflammatory cells (Fig. 3B).

Table I. Characteristics of patients included in the study.

Patients characteristics	Chronic HCV patients Mean±SD	HCV fibrotic patients Mean±SD
Gender	Male= 20, Female= 20	Male= 20, Female= 20
Age 31-50 years	41.87±5.59	42.27±5.40
Viral load (IU/L)	$3.3 \pm 2.52 \times 10^5$	$4.08 \pm 2.60 \times 10^5$
ALT (U/L)	78.83±50.01	79.32±34.54
ALP (U/L)	137.77±60.20	159.92±73.94
Total bilirubin (mg/dl)	2.72±1.41	2.82±1.67

ELISA of IL17A and protein-protein interactions

ELISA analysis revealed increased IL-17A expression in both group of patients. The mean serum level of IL17A (Table II) was higher; 75.26 ± 21.22 pg/ml (range: 36.2-117.54 pg/ml) in the fibrotic group than the chronic group; 45.03 ± 6.20 pg/ml (range: 29.76-56.89 pg/ml) with a statistically significant difference ($P < .01$). We observed a non-significant ($P > .05$) correlation between ALT, ALP and total bilirubin versus IL-17A (Table II). There was strong positive correlation (chronic, $r = 0.88$; fibrotic $r = 0.97$) between level of IL17A with age. Similarly, a strong positive correlation ($r = 0.75, 0.93$) between IL-17A and viral load was also observed, which was statistically highly significant ($P < .01$).

Because IL-17A has the potential to influence the expression of other genes, especially inflammatory, gene network analysis by STRING database online tool showed

an overall interaction (Fig. 1) profile of IL-17A with the provided list of gene IDs. The input list had various genes with different functions which are related to different behaviors in biological pathways. However, network generated by GeneMANIA (Fig. 2) showed IL-17A inclination of interaction (particularly co-expression) with only a few genes i.e., IL-6, NFKB1A, CXCL1, CXCL6, CXCL8 etc. This result suggests a possible role for IL-17A in regulating the expression of chemokines.

Table II. Correlation between IL-17 levels and various patient characteristics.

	Chronic		Fibrotic	
IL-17A (pg/ml) Mean±SD	44.97±6.28		75.26 ± 21.22	
Range (pg/ml)	(29.76-56.89)		(36.2-117.54)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	0.88	< .01	0.97	< .01
Viral load	0.75	< .01	0.93	< .01
ALT	-0.14	> 0.05	0.04	> 0.05
ALP	-0.16	> 0.05	0.09	> 0.05
Total bilirubin	-0.02	> 0.05	-0.11	> 0.05

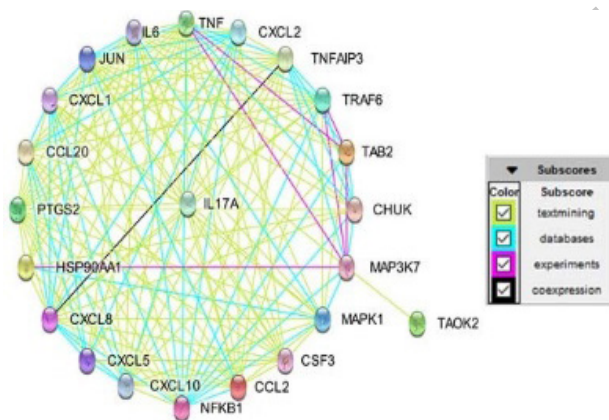


Fig. 1. IL-17A gene interaction network using the STRING database.

Liver of HCV infected patients

In the normal control group, lobular architecture with normal distribution of hepatocytes, central vein and portal triad and radiating hepatic cords exhibited by H and E and Masson's trichrome staining (Fig. 3A, C). In the fibrotic group enlarged central veins with hemorrhage and congestion of centrilobular architecture (Fig. 3B, D). Huge infiltration of inflammatory cells was observed radiating from central vein to portal triad representing bridging inflammation. Profound damage to liver tissue was validated by the existence of centrilobular necrosis,

immense hemorrhage and noticeable neutrophil infiltration (Fig. 3B). Staining with Masson's trichrome showed bridging fibrosis with intense feature of fibrous bands expanding from one portal triad to another alongside distortion in the normal hepatic architecture (Fig. 3D).

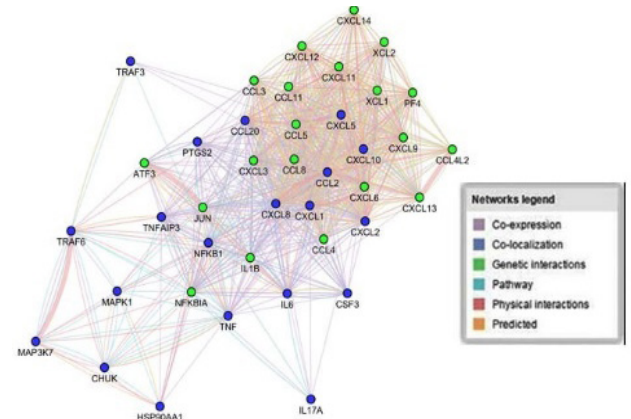


Fig. 2. IL-17A gene interaction network using the GeneMANIA.

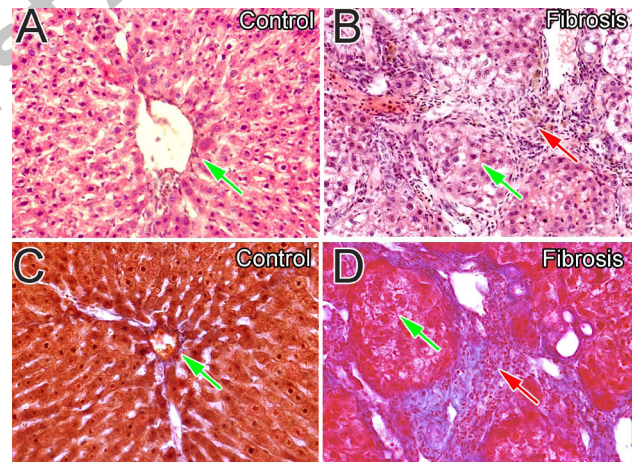


Fig. 3. H&E and Masson's trichrome staining of liver tissues of HCV infected patients. A, Stained with H/E, the control group with no sign of any abnormality; B, H/E shows cords of fibrosis with mixed infiltration of inflammatory cells; C, Masson's trichrome staining normal control group with distinct shaped nuclei of hepatocytes and their arrangement in cords; D, Masson's trichrome staining showing extensive fibrosis. The shapes of hepatic lobes and lobules were distorted in the fibrosis group.

Immunohistochemistry of Kupffer cells (KCs)

In Figure 5A, an even and uniform distribution of KCs throughout the hepatic lobules was present as normally these cells are present in quiescent stage in the sinusoidal spaces and also in the space of Disse. The existence of KCs was even more predominant in the merger of DAPI and

CD68 antibody which identifies the clear morphological distribution of KCs as shown in Figure 5C. In the hepatic lobules of fibrotic and cirrhotic patients, most of the KCs adopted spindle like shape in the lining of sinusoids (Fig. 5D) while in the portal region it appears as round cells (Fig. 5E) as evident from DAPI staining. We have used double approach of merging the KCs with DAPI to find their architecture in the hepatic lobules and in the perisinusoidal space (Fig. 5F). Compared to the normal control group, an increased number of KCs was identified in the livers of HCV patients, and these cells seem to concentrate in the scars during the course of advanced fibrosis (Fig. 5F). Furthermore, it is noticed that numerous types of scar-associated KCs were found different in appearance from normal hepatic macrophages. Remarkably, compared to normal control, very less CD68 positive stained cells were found in the parenchymal areas of fibrotic and cirrhotic liver (Fig. 5D).

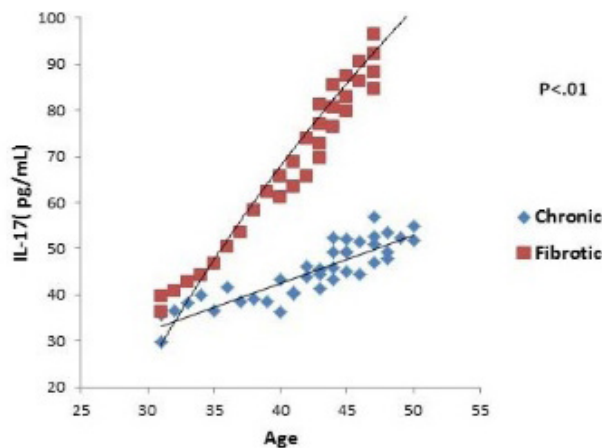


Fig. 4. The correlation of IL-17A serum levels with age in chronic vs. fibrotic patients.

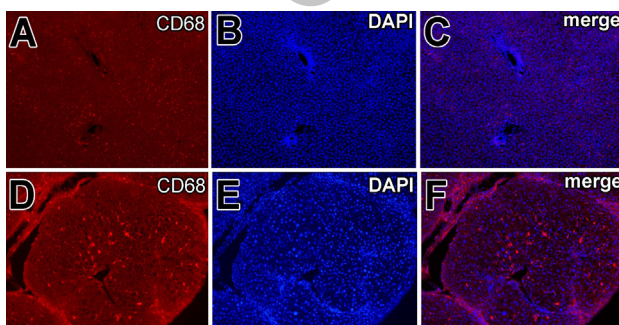


Fig. 5. Localization and expression of macrophages in normal and fibrotic liver. Reduced the number of CD68+ cells: Double fluorescence for CD68 and DAPI in normal liver (A, B, C) and in the fibrotic liver showed numerous CD68+ cells along the sinusoids around the central vein (D, E, F).

Immunostaining of alpha (α)-smooth muscle actin

In the fibrotic group, huge number of α -SMA positive HSCs was identified in the sinusoids and were confined in to the hepatic lobules mostly in the peripheral zones (Fig. 6D). Deposition of collagen and activation of HSCs in association with pericentral necrosis led to wound healing response in cirrhotic patients. Figure 6A demonstrates expression of collagen irradiating from portal area making fibrous scars enclosed nodular formation.

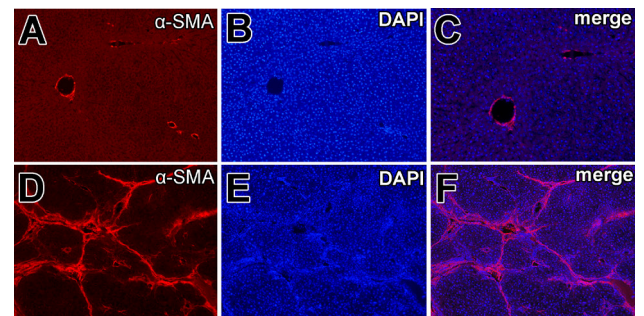


Fig. 6. Immunohistochemistry of alpha SMA in the liver of HCV positive patients. Normal control group with alpha SMA, DAPI and merge (A, B, C) While in the cirrhotic group there is huge expression of alpha SMA making bridging fibrosis (D, E, F).

The biopsies specimen of HCV patients showed strong and diffuse immunostained, α -SMA-positive HSCs (Fig. 6F). In the expanding septa and in the perisinusoidal spaces many HSCs seems moving and crawling with dilated morphological features confined to the regenerative plates (Fig. 6D), which is also confirmed from DAPI staining (Fig. 6E). HSCs seem diverse in size and shape, even though most of them with stretched elongated cytoplasmic processes along the endothelial lining (Fig. 6F). Normal control group showed confined expression limited to central vein and around the vicinity of portal region was observed (Fig. 6A-C). It showed that staining of α -SMA in the parenchymal and portal region of cirrhotic liver compared to normal healthy liver (Fig. 6A and D).

Expression of HepPar1, Arginase-1 and Ki-67

HepPar1 staining was noticed in the differentiated hepatocytes surrounded by cirrhotic tissue and in the nodules of liver parenchyma. Variable staining pattern was identified, the cytoplasm of some hepatocytes showed brown punctate while some have more intense dark brown appearance of cytoplasm (Fig. 7C, D), interestingly some showed granular appearance. Also trace of HepPar1 staining was identified in some places at the vicinity of tissue in the normal control (Fig. 7A, B), while rest of the structure showed slightly darker staining reveal

heterogeneous array from small to large shape of HepPar1-positive cells (Fig. 7C, D). No staining reactivity was found for KCs, sinusoidal endothelial cells.

Arginase-1 (Arg-1) is mostly found in the liver and catalyzes the conversion from arginine to ornithine in the urea cycle. Immunohistochemistry of Arg-1 indicated diffuse cytoplasmic staining in both tumorous and non-tumorous hepatocytes (Fig. 7E, F). In the fibrosis and cirrhotic liver tissues showed diffuse cytoplasmic pattern with distinct nuclear reactivity (Fig. 7C, D). In the normal liver sections, Arg-1 staining showed robust and diffused reactivity cytoplasmic content of hepatocytes all over the lobule (Fig. 7E, F).

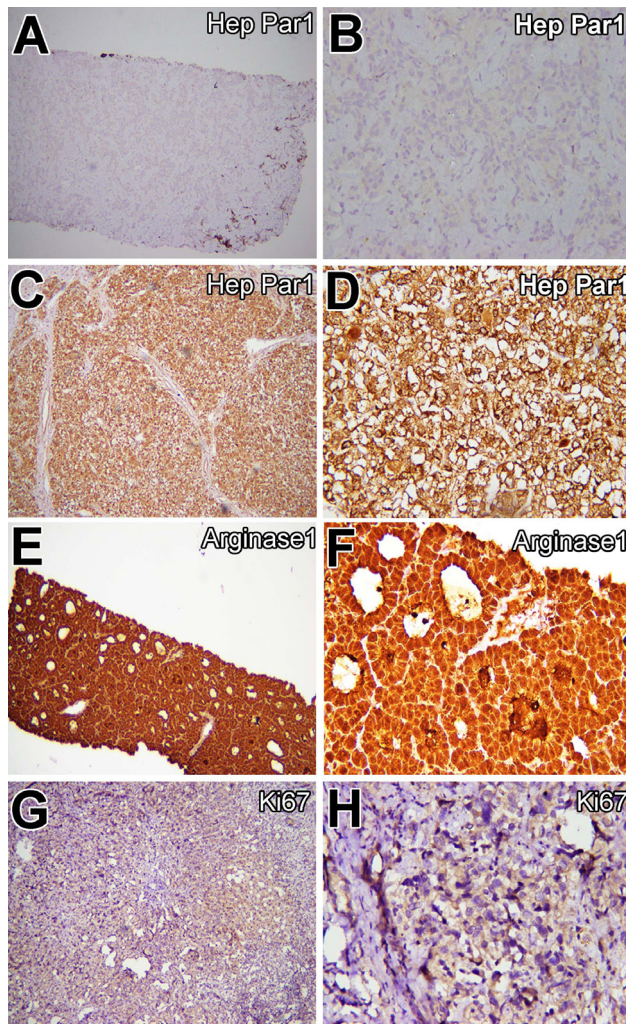


Fig. 7. Immunohistochemical labeling for HepPar1 (A, B) in normal and cirrhotic liver (C, D) in liver biopsies of HCV patients. Arginase-1 staining (E, F), Ki-67 (G, H) in the portal tracts and lobules in HCV infection exhibit a higher expression compared with samples normal liver.

The expression of proliferative marker Ki67 was identified in the liver tissue biopsies of cirrhotic patients (Fig. 7G, H). The immunoreactivity of Ki67 was mainly localized to the nuclear membrane and nucleolus and their expression was assessed in terms of positively stained cells by the antibody. While in the normal control liver limited number of immunostained positive cells was identified. The increase in the number of Ki67 immunostained cells was observed in patients with cirrhosis with densely stained nuclear membrane of the hepatocytes at the vicinity of fibrotic band (Fig. 7H) compared to fibrosis patients.

DISCUSSION

Inflammatory molecules have a critical role in the regulation of the early immune response, albeit a successful outcome depends on a balanced immune response in diseases including CLDs. IL-17 for example, may promote hepatic fibrogenesis and facilitate the development of HCC. On the other hand, IL-22 exhibits a protective role during the development of fibrosis (Hammerich and Tacke, 2014). The current study was conducted with an aim to measure a key inflammatory marker i.e., IL-17A in chronic and fibrotic HCV positive patients belonging to the southern districts of KPK province of Pakistan. In our results, serum IL-17A levels showed a positive correlation ($P < .001$) with age in both groups of patients and had statistically significant association with other factors. Previously, expression of some biomarkers including IL-17 was shown to be associated with HCV severity in HCC (Aboushousha *et al.*, 2021). In case of another liver-targeting pathogen, the hepatitis B virus (HBV), significantly higher serum levels of IL-17 were also associated with chronic progression to HCC. Similarly, serum IL-17 expression in chronic HBV infection was increased (Tian *et al.*, 2019), and associated with the degree of liver fibrosis (Du *et al.*, 2013). Increased serum values of IL-17A in a group of chronic HCV patients also positively correlated with the degree of liver fibrosis (Gomaa *et al.*, 2019), and an aggravated clinical state progressing from HCV infection towards HCC through cirrhosis (Hammad *et al.*, 2013). Moreover, serum IL-17 levels were significantly ($P < .001$) higher in chronic HCV and cirrhotic patients showing positive correlation with the grade of inflammation and the stage of fibrosis (Hassan *et al.*, 2014). In addition to the chronic HCV infection, the presence of some other factors could contribute to the accelerated progression of CLD, such as alcohol consumption, obesity and older age etc. (Younossi *et al.*, 2016). We herein also observed similar results and serum IL-17A levels significantly correlated with age in both chronic and fibrotic patients. Ouyang *et al.* (2011) reported a significant increase in IL-

IL-17-producing CD4⁺ T cells and mRNA expression in aged people compared to young healthy individuals (Ouyang *et al.*, 2011). Similarly, the distribution of IL-17-producing cells (Th17 cells) was significantly ($P < .001$) increased in the tumors of HCC patients, suggesting that accumulation of IL-17-producing cells may support tumor progression (Zhang *et al.*, 2009). A systematic review concluded that in case of cirrhosis (due to HCV infection), old age, heavy alcohol intake and the male gender may influence rapid progression of the disease (Freeman *et al.*, 2001). The significant increased serum levels of IL-17 and its gene in relation to grades of inflammation and different stages of hepatic fibrosis (Kassim *et al.*, 2017), thus highlights a possible new inflammation-associated mechanism in CLDs, which could lead to the discovery of a suitable and precise diagnostic and therapeutic tool in clinical practice.

The pro-inflammatory properties of IL-17 during the pathogenesis of various diseases are mostly related to its capability to recruit the immune cells, and IL-17 achieves this effect in large part due to the induction of new gene expression (de Morales *et al.*, 2020; Onishi and Gaffen, 2010). This pivotal role of IL-17/IL-17A alone or in synergism with other cytokines/chemokines/proteins in driving tissue pathology of various tissues including the liver in an immune-mediated fashion in different disease conditions have been evaluated and reported (Hartupce *et al.*, 2007; Noack *et al.*, 2019; Harley *et al.*, 2014; Liao *et al.*, 2019). By analyzing the gene-gene interactions of IL-17A with the help of STRING and GeneMania online database tools, we observed that IL-17A (Figs. 3 and 4) interacts with various other genes- the majority of which are chemokines. Gene interactions participate in nearly all ongoing biological processes within living organisms. Many studies have shown that any disruption in the normal pattern of gene-interaction pathways in humans can be indicative of a disease condition, or may have a fundamental impact on the progression of a particular pathological state including infectious diseases (Kuzmanov and Emili, 2013).

Pakistan suffers from the second largest numbers of HCV infections and CLDs accounts for the fifth most common reasons behind mortality and morbidity across the country, thereby earning the nickname “the cirrhotic state”. In Pakistan approximately 68% of HCC is caused by HCV related cirrhosis (Parkash and Hamid, 2016; Abbas and Abbas, 2020). Pakistan has pledged to the aspiring objective of eradicating HCV by 2030. A disturbing reality about the HCV prevalence in Pakistan is that over the last three decades, the high rates of infections are strikingly persistent with no evidence for a decline (Mahmud *et al.*, 2019), making it almost impossible to achieve the goal in the near future. Pakistan has HCV genotype 3 (subtype

3a) as the most prevalent genotype across the country. Increasing clinical and experimental data show that HCV genotype 3 is involved in a more rapid advancement of CLDs i.e., accelerated fibrosis progression rate, increased cirrhosis and higher incidence of HCC (Ampuero *et al.*, 2014; Goossens and Negro, 2014), by producing cytopathic effects during the damage of the liver (Kumar *et al.*, 2002; Rubbia-brandt *et al.*, 2000). As the number of unidentified chronic HCV infections increase (due to under-screening), one of the long term consequences could be an escalation in the numbers of cirrhotic patients and HCC across Pakistan which will put a direct and enormous pressure on the underprivileged healthcare system of the country.

Liver cirrhosis and fibrosis cause basic fundamental changes in hepatic architecture induced distortion through formation of bridging fibrous septae and necrosis with simultaneous regenerative nodules formation. There are various etiological factors for development of CLD having cirrhotic histology. In the current study, it is also identified that HCV-induced CLD causes enhanced accumulation of fibrous bands. Their first evidence is the activation of α -SMA, HSCs and their transmigration from perisinusoidal spaces to the site of injury compared to the normal liver tissues. Our finding regarding the activation of HSCs in chronic HCV infection compared to normal liver are in support with previously reported findings (Schmitt-Gräf *et al.*, 1991; Yamaoka *et al.*, 1993; Guido *et al.*, 1996; Ueno *et al.*, 1997; Martinelli *et al.*, 2004; Xaus *et al.*, 2000). The magnitude in the increase expression of HSCs in the chronic HCV liver disease compared to normal liver depends on the number of samples and severity of infection as identified by immunohistochemistry techniques (Yamaoka *et al.*, 1993; Guido *et al.*, 1996; Martinelli *et al.*, 2004; Xaus *et al.*, 2000). We found enhanced α -SMA-positive cells in the liver of HCV patients compared to normal livers.

A decrease of KCs was identified in the lobular area of normal liver while slender shaped cells located in the fibrous band in chronic HCV liver. In some liver tissues of HCV positive patients a decrease of KCs might be due to programmed cell death during infection (Xaus *et al.*, 2000). Moreover, HCV infection can stimulate liver macrophages which can release pro-inflammatory mediators and an elevation of IL-10 in the serum. The association of Ki67 expression in HCV positive liver tissues showed strong positivity for nuclear membrane of hepatocytes. Other studies have pointed out that cirrhosis association with dysplastic changes found negative for Ki67. In our study, overall, an enhanced Ki67 labeling index was observed in the chronic liver injury of liver (Mocanu *et al.*, 2012).

During analysis we found that liver of fibrotic and cirrhotic patients represented predominant expression

of Arg-1 in contrast to other normal tissues. Both in normal and chronic HCV liver Arg-1 exhibited verbose cytoplasmic expression together with irregular nuclear reactivity. It showed the sensitivity and specificity of Arg-1 for cirrhotic liver samples and the same reactivity was also identified using HepPar1 (Fan *et al.*, 2003). Other studies also indicated their reactivity in both cases which is supportive for our work (de Gonzalez *et al.*, 2015).

As the presence of chronic inflammation is associated with the development of fibrosis, cirrhosis, HCC etc., the severity of liver fibrosis is a crucial factor to be analyzed before deciding on the treatment options for a patient (Hammerich *et al.*, 2011). We, herein propose further evaluation of IL-17A as a possible biomarker of ongoing inflammation and a prognostic marker for liver fibrosis/cirrhosis in patients with chronic HCV across Pakistan. Moreover, this study uncovers the possibility of blocking inflammation-associated biomolecules i.e., IL-17A in chronic HCV patients as a “prevention first” strategy which may be a suitable option to control inflammation in CHC ultimately hindering progression to cirrhosis and HCC. IL-17 targeted therapy from preclinical and clinical trials has shown promise in several autoimmune conditions (Zhang *et al.*, 2015). Secukinumab, for example, is a fully human monoclonal antibody against IL-17A which has already received global approval for the treatment of patients with psoriasis (Sanford and Mackeage, 2015). Fortunately, Secukinumab therapy exhibited a highly promising efficacy in treating HCV patients; only 1 out of 14 (7.1%) HCV patients showed enhanced replication of HCV and no virus reactivation occurred in patients receiving antiviral prophylaxis (Chiu *et al.*, 2018). We can assume that the future use of Secukinumab might directly be utilized alongside other available DAAs globally including Pakistan to control endemic HCV.

CONCLUSION

We found that IL-17A was altered and observed an increase with age. The abnormal levels of IL-17A with increasing trend in aged people could be due to a decrease in the protective immunity and the persistence of inflammation in both chronic and fibrotic HCV patients. Gene network analysis revealed the inclination of IL-17A through co-expression towards chemokines. This infers the potential of IL-17A in recruiting specific types of cells (i.e., neutrophils) to the site of injury in the liver, thus propagating inflammation. The possible role of IL-17A in chronic inflammation in the context of CHC (viral) infection could serve as the futuristic interventional therapeutic target to block the progression of CLDs into HCC. Furthermore, IHC analysis provided an insight

into the characterization of distorted hepatic structure, reinforcing the importance of diagnostic markers in the diagnosis of advance CLDs including cirrhosis and HCC.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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