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Immunohistochemical Study of Hepatic Progenitor Cells by c-Kit (CD117) in Chronic Hepatitis C Viral Liver Disease

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Abstract: Egypt has the highest prevalence of hepatitis C virus (HCV) in the world, ranging from 6% to 28%. Liver progenitor cells represent a key area in the development of new therapeutic strategies for liver disease. We aimed in this study to evaluate the immunohistochemical study of hepatic progenitor cells by c-Kit (CD117) with statistical evaluation and correlation with the clinical data & pathological features in HCV liver disease. The current study includes sixty cases of chronic hepatitis C collected from Pathology department, Faculty of medicine, Cairo University, during the period from February 2013 to March 2014, studied histologically and immunohistochemically for c-Kit (CD117) expression. *Results showed that:* Hepatic progenitor cells (HPC) are well correlated with necroinflammatory grades and fibrosis stages. c-kit positive HPC were found in 48/60 studied HCV cases (80%). However, some HPC ductular reaction in H&E stain didn't take c-kit immunostain. Values of c-kit positive HPC were in range (0-18) in non-cirrhotic HCV. Values were in range (18-50) in cirrhotic HCV with minimal cut eighteen cells. A statistically significant directly proportional relationship was detected between hepatocellular carcinoma & HPC with minimal cutoff twenty cells. A statistically significant direct proportional relationship was detected between hepatocellular carcinoma (HCC) & HPC with minimal cutoff twenty cells. A statistically significant relationship was detected between HPC number & cholestasis. Therefore, a close search and reporting of HPC is recommended as it could be useful in predicting the progression in HCV patients. So, clear and precise delineation of markers specific to HPC is highly recommended and use of more than one stem cell marker will be more reliable to define HPC.

Key words: Hepatitis C virus infection (HCV) · Hepatic progenitor cells (HPC) · Hepatocellular carcinoma (HCC) · c-Kit (CD117)

INTRODUCTION

Egypt has the highest prevalence of hepatitis C viral (HCV) infection in the world, ranging from 6% to 28% with an average of approximately 15% in the general population [1]. Hepatic progenitor cells (HPC) are small, narrow cells with a basophilic cytoplasm and an oval nucleus and they appear after all kinds of liver damage. These cells are bipotential and have the ability to differentiate into either hepatocytes or biliary epithelial cells. c-Kit is considered to be a determinant marker for these cells [2]. Liver progenitor cells represent a key area in the development of new therapeutic strategies for liver disease. These cells represent a "stand-by" stem cell compartment that can be activated and driven to differentiate into mature lineages through modulation of cytokines and growth factors. The shortage of liver donors and the well-known limits of liver transplantation underscore the need and the possibility of applying a stem cell therapy to end-stage chronic hepatic diseases and to acute massive liver injury [3].

The aim of this work was the immunohistochemical study of hepatic progenitor cells by c-Kit (CD117) with statistical evaluation and correlation with the clinical data & pathological features in HCV liver disease.

MATERIALS AND METHODS

This retrospective study was conducted on sixty cases of chronic hepatitis C cases collected from Pathology department, Faculty of medicine, Cairo University, during the period from February 2013 to March 2014. The cases were chosen at different stages of the disease (From early to advanced and complicated) taking into consideration the available remaining tissue in paraffin blocks.

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The protocol of this study was approved by the Ethics committee of the Faculty of Medicine, Cairo University for use of patients' specimens for research purposes.

These cases were proved clinically and serologically to be positive for hepatitis C virus. Age, sex and clinical data were taken from the accompanying sheets. Twenty cases were hepatectomy specimens, thirty-nine were core biopsies and one wedge biopsy. Concerning the chronic hepatitis C cases complicated with hepatocellular carcinoma, sections were taken mainly from the adjacent cirrhotic liver tissue with part of the tumour or any neoplasia related changes.

Histopathological Procedure: Tissue sections from the paraffin blocks were cut at 4 micron thickness and Haematoxylin and Eosin for histopathological diagnosis and with Masson Trichrome for assessment of fibrosis.

Sections were examined microscopically to evaluate the following features:

- The degree of fibrosis whether periportal, septal or bridging (porto-central or porto-portal) and the presence or absence of architectural distorsion with or without cirrhosis.
- Presence of neoplasia related changes and whether these were large or small cell change.
- Presence of hepatocellular carcinoma.
- Presence and grading of steatosis according to Brunt score [4].
- Presence or absence of cholestasis.
- Ishak modification for hepatic activity index (HAI) and for staging of liver fibrosis in chronic hepatitis [5]. For statistical purposes; scores of 0 or 1 were assigned to cases of mild liver damage; scores of 2 to 5 to moderate cases; and scores of 6 to 12 to marked necroinflammation cases [6].
- METAVIR classification for hepatitis C liver disease for the evaluation of histological activity and staging [7].

Immunohistochemical Procedure: Sections (3 microns thick) were prepared from (non-exhausted) paraffin blocks on charged glass slides, manually treated for antigen retrieval and then treated with antibodies using avidin-biotin peroxidase technique. DAB was used as a substrate and chromogen. Hematoxylin was used as a counter stain.

Application of Primary Antibodies: The antibody was used in the appropriate dilution, using primary antibody diluent (Genemed, USA). The c-kit (CD 117) anti-Human rabbit monoclonal antibody (Genemed, USA) was used in a dilution range of 1:50.

Control Slides: Positive and negative control slides were included within each session. The negative control was performed by omitting the primary antibody and the positive control employed were sections taken from a known case of c-kit positive gastrointestinal stromal tumour and a case of breast fibroadenoma.

Immunohistochemical Scoring: Cells were scored when they satisfied the morphological criteria and highlighted by cytoplasmic staining for c-kit. The number of oval cells was subsequently assessed in each biopsy, by calculating the average number of hepatic oval cells per high-power field (HPF) based on the count of hepatic progenitor cells in three non-overlapping HPFs, using a 40× objective giving a field diameter of 0.5 mm [8]. To avoid artificial effects, cells in areas with necrosis, poor morphology or at the margins of sections were not counted.

Statistical Analysis: Data management and analysis were performed using Statistical Package for Social Sciences (SPSS) vs. 21. Numerical data were summarized using means and standard deviations or medians and ranges. Categorical data were summarized as percentages. Comparisons between the two groups with respect to normally distributed numeric variables were done using the t-test. None normally distributed numeric variables were compared by Mann-Whitney test. For categorical variables, differences were analyzed with χ^2 (chi square) test and Fisher's exact test when appropriate. Correlations among HPC and other parameters were determined by using Sperman rho test. All p-values are two-sided. P-values ≤ 0.05 were considered significant.

RESULTS

Clinicopathological Results: Sixty cases were enrolled, most of them were males (48/60), representing, 80% of cases; with male to female ratio 4:1. All cases were in adult middle age group (23-59) with mean age 45.43±SD 10.046. Mean age in males was 45.66±SD 10.184; while mean age in females was 44.45±SD 9.842.

Regarding portal inflammation, score 3 portal inflammation was the most frequent; being seen in 23 cases (38.3% of studied cases). While score 2 was the most frequent in both interphase hepatitis and lobular inflammation; being seen in 21 cases (21/60, 35% of studied cases).

Diversity in Ishak hepatic activity index was noted. Hepatic activity index grade 6 & grade 12 were the most frequent grades (n=10 cases, 16.7% each). Grade 12 was exclusively found among cirrhotic patients (10/20 cirrhotic cases). There were no cases with grade 13-18 (no confluent necrosis) or grade 1. Regards Ishak fibrosis staging; most cases (35%) were cirrhotic (stage 6). Most cases (38.3%) were of METAVIR activity index A3. Most cases (35%) were of stage F4 in METAVIR fibrosis staging.

Neoplasia related changes; including large cell change & small cell change were found in 7 cases (5 cases with HCC on top of cirrhosis & 2 cases with end stage liver cirrhosis). HCC represented 11.7% of total cases; though hepatocellular carcinoma cases represented 35% of hepatectomy specimens (7/20). Steatosis was detected in 48.3% of studied cases. The most frequent grades in all studied cases were of steatosis grade zero (51.7%) & one (28.3%). Cholestasis was found in only nine cases, representing 15% of cases (four of them were with HCC).

Table 1: Correlation between age and HCV histopathologic changes:

		Age
Portal inflammation	r	.594
	p	<0.001
	N	58
Interface hepatitis	r	.332
	p	.011
	N	58
Lobular inflammation	r	.314
	p	.017
	N	58
Presence of hepatocellular carcinoma	r	.280
	p	.034
	N	58
Hepatic activity index GRADE	r	.433
	p	.001
	N	58
Hepatic activity index STAGE	r	.558
	p	<0.001
	N	58

- Spearman's rho; r (range), p (probability) & N (number).

• A statistically significant directly proportional relationship was detected between patients' age & portal inflammation, interface hepatitis and lobular inflammation.

• A statistically significant directly proportional relationship was detected between age & HAI grade and stage (p?0.001).

• A statistically significant relationship could be detected between patients' age & the presence of HCC.

Table 2: Correlation between HCC & HCV histopathologic changes:

	Hepatocellular carcinoma			
	Median	Minimum	Maximum	p value
Portal inflammation	4	4	4	<0.001
Interface hepatitis	4	3	4	<0.001
Lobular inflammation	4	3	4	<0.001
HAI grade	12	10	12	<0.001
HAI stage	6	6	6	<0.001

• A statistically significant directly proportional relationship was detected between HCC & portal inflammation, interface hepatitis, lobular inflammation, as well as HAI grade and stage.

• No HPC were seen just around hepatocellular carcinomatous nodules. HPC were found in the desmoplastic areas away from the carcinomatous cells (Figure 6). Also seen forming ductules & cords around the cirrhotic nodules with or without neoplasia related changes.

HCC was exclusively found in male gender in studied cases, occurring in 14.6% of male gender with HCV, with mean age 53±SD 2.098.

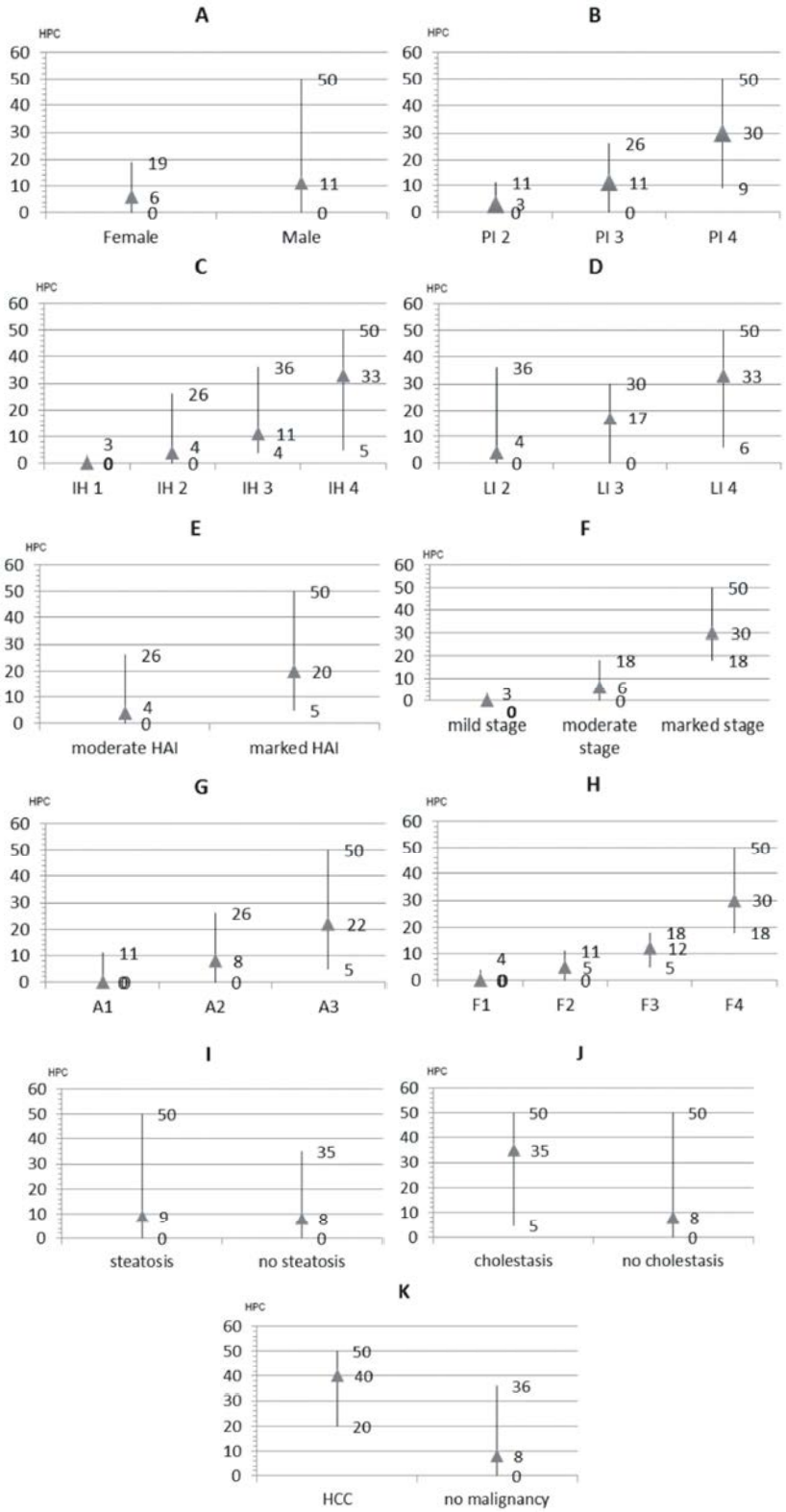
Table 3: Correlation between HCC & steatosis and cholestasis:

		Presence of malignancy			
		No	Yes	Total	P value
Non steatotic	Count	30	1	31	
	Percentage	96.8%	3.2%	100.0%	
Steatotic	Count	23	6	29	
	Percentage	79.3%	20.7%	100.0%	
Total	Count	53	7	60	
	Percentage	88.3%	11.7%	100.0%	0.04
Non cholestatic	Count	48	3	51	
	Percentage	94.1%	5.9%	100.0%	
Cholestatic	Count	5	4	9	
	Percentage	55.6%	44.4%	100.0%	
Total	Count	53	7	60	
	Percentage	88.3%	11.7%	100.0%	0.01

HCC occurred in 20.7% of HCV cases with steatosis and 44.4%with cholestasis. Hepatocellular carcinoma was found in 30.4% in METAVIR A3 grade, while being absent in A0, A1 and A2.In this study, HCC was uniquely found in cirrhotic cases. It was found in 33.3% in METAVIR F4 stage, while being absent in F0, F1, F2 and F3

Immunohistochemical HPC Detection Results: HPC were observed in and around portal tracts and along the fibrous septa, mostly in small clusters, cords or forming ductules. Ductular reaction was so evident in cirrhotic cases (Figure 2). No intra-acinar HPC were observed.

c-kit cytoplasmic positive HPC were found in 48 cases out of 60 studied HCV cases (80%). No c kit membranous c stain was observed in HPC. The twelve c kit negative cases showed mild grade & stage with no HPC noticed as regards morphology in H&E stain. However, some HPC ductular reaction within the positive cirrhotic cases in H&E stain didn't take c-kit immunostain (Figure 8). A statistically significant directly proportionate relationship was detected between hepatic progenitor cells number HPC & age (P value >0.001).



Graph 1: Correlation between Hepatic progenitor cells (HPC) &:A) gender, B) Portal inflammation (PI), C) Interface hepatitis (IH), D) Lobular inflammation (LI), E) Hepatic activity index (HAI), F) Ishak stage, G) METAVIR activity (A), H) METAVIR fibrosis (F), I) Steatosis, J) Cholestasis, K) Hepatocellular carcinoma (HCC).

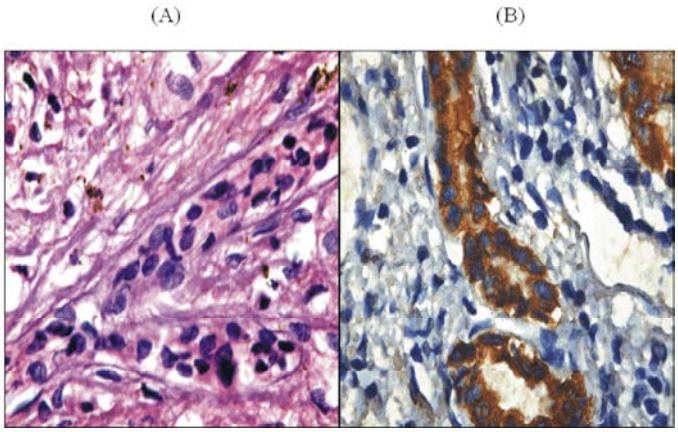


Fig. 1: A) HPC with oval shape exhibiting a high nuclear/cytoplasmic ratio and an ovoid nucleus ((H&E oil immersion x1000). B) HPC with c-kit positive cytoplasmic stain (c-kit immunostain, oil immersion x1000)

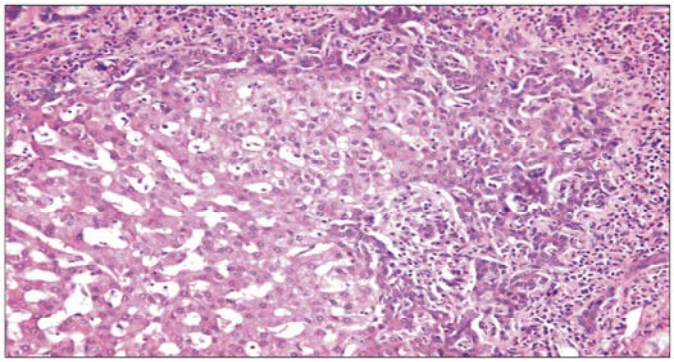


Fig. 2: HPC crown a cirrhotic nodule with cord and ductular forms (H&E x200)

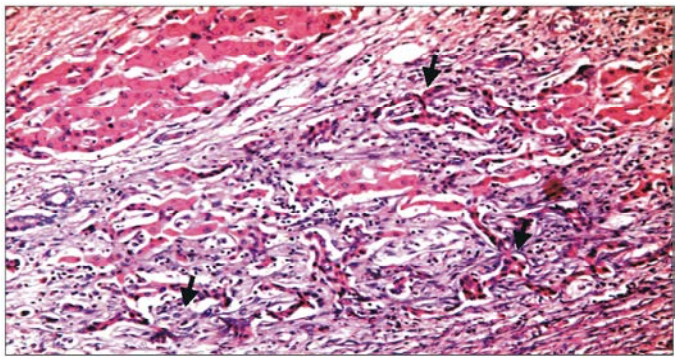


Fig. 3: Intermediate hepatocytes admixed with HPC (black arrows) and dense inflammatory cells (H&E x200)

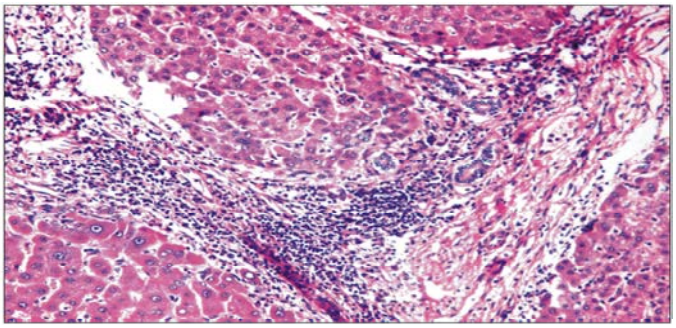


Fig. 4: Large neoplasia related change with prominent ductular reaction and dense portal inflammation (H&E x100)

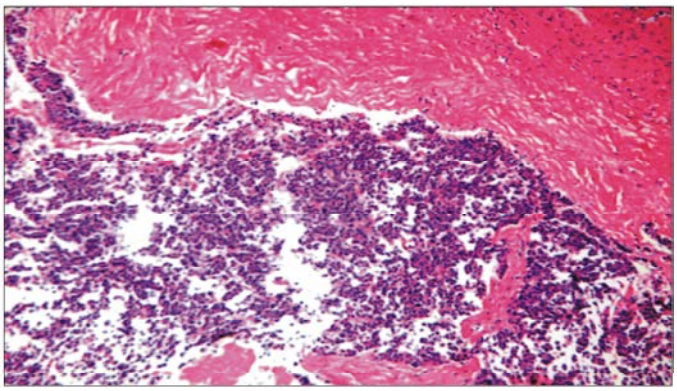


Fig. 5: HCC with desmoplasia devoid of HPC (H&E x200)

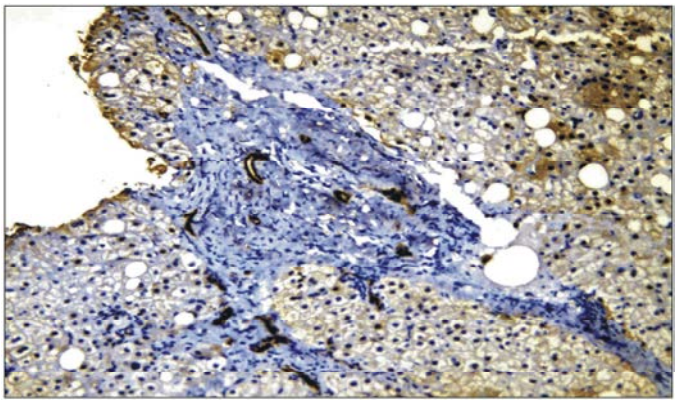


Fig. 6: HPC form cords and ductules within the fibrous tissue (c-kit immunostain x200)

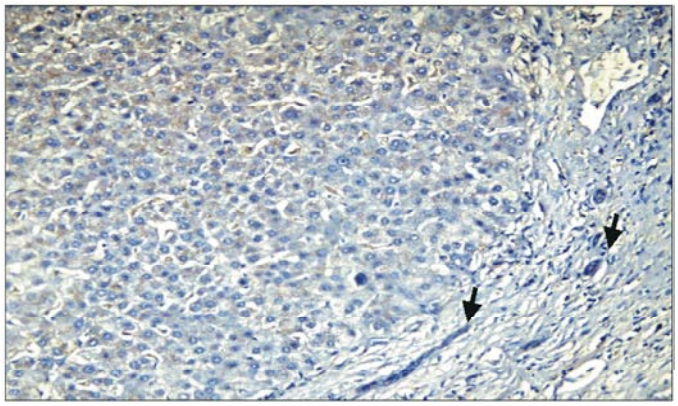


Fig. 7: HPC in the form of ductular structure with c-kit negative stain (black arrows) (c-kit immunostain x100).

As this study includes cases of liver core biopsies with different stages of the disease (From early to advanced and complicated) as well as hepatectomy specimens, the result data were not normally distributed; hence the hepatic progenitor cells were represented by minimum, maximum and median Δ values.

No statistically significant relationship could be detected between HPC and gender (P value 0.061). A statistically significant directly proportionate relationship was detected between HPC& portal inflammation, interface hepatitis, lobular inflammation, Ishak HAI, Ishak stage of fibrosis, METAVIR activity and stage of fibrosis. Values were in range (0-18) in non-cirrhotic HCV. Values were in range (18-50) in cirrhotic HCV with minimal number eighteen cells. No statistically significant relationship could be detected between HPC and steatosis (P value 0.898). A statistically significant relationship was detected between HPC & cholestasis as well as hepatocellular carcinoma & HPC with minimal number twenty cells.

DISCUSSION

Chronic human liver diseases are characterized by continuous liver damage and hepatocyte loss, with subsequent activation of the progenitor cell compartment [9]. Considerable interest has been devoted to the HPC, as tumors showing HPC features have a worse prognosis and a higher recurrence rate compared to tumors lacking these characteristics [2].

In the present study, the male predominance is clear with male gender representing 80% of cases; with male to female ratio 4:1. All cases were in adult middle age group (23-59) with mean age 45.43± SD 10.046.Mean age in males was 45.66± SD 10.184; while mean age in females was 44.45± SD 9.842. These results are in concordance with results of the Egyptian Demographic Health Survey in 2009. HCV infection was more frequent in young and middle-aged adults and in males than females [1].

Similarity between Ishak & METAVIR fibrosis staging systems regarding frequency results was noticed; however a discrepancy in necroinflammatory grade between the two systems was noted. Marked necroinflammation representing 81.3% of cases; while in METAVIR grade, marked necroinflammation represented 38.3% of cases. This goes with what stated by Rozario and Ramakrishna [10] that concordance between Ishak and METAVIR scoring systems is good for necroinflammatory change and excellent for fibrotic change.

Among the histopathological findings in HCV, the occurrence of atypical hepatocytes is significantly correlated with the risk of developing HCC together with progressive liver fibrosis; it would be meaningful to perform liver biopsy in patients with progression of liver fibrosis [11]. Neoplasia related changes; including large cell change & small cell change were found in seven cases (11.6%) (Five cases with HCC on top of cirrhosis & two cases with end stage liver cirrhosis)

Demographic and epidemiological factors are linked to fibrosis progression in chronic HCV infection. A statistically significant directly proportional relationship was detected between patients' age and portal inflammation. A statistically significant relationship could be detected between patients' age & interface hepatitis and lobular inflammation. These were similar to what stated by Ryder [13].

HCC is a known lethal complication of HCV. Liver cancer constitutes 13% of all cancers in Egypt and is considered the second most frequent cancer in males [14]. HCC was found in seven cases with mean age 53±SD 2.098. Similarly, Guimei *et al.* [15] stated that mean age of cirrhosis & HCC patients was 53.67 ±SD 7.4. Baddour *et al*. [16] documented that the patient age ranges were 47-70 (56.1±7.7) years in the cirrhosis & HCC group. That may be explained by the fact that liver cancer is generally attributed to HCV. Chronic HCV most probably leads to carcinogenesis after 10-30 years following infection.

HCC was exclusively found in male gender in studied cases, occurring in 14.6% of male gender with HCV. Kumar *et al*. [17] found that 84% of the HCC patients were male. Yeh and Chen [18] reported molecular mechanisms underlying the carcinogenic effect of both sex hormones. Knockout of androgen receptor (AR) expression in hepatocytes delayed the development of HCC. Estrogen can protect hepatocytes from malignant transformation via downregulation of IL-6 release from Kupffer cells. This demonstrated that the gender disparity of HCC is attributed by both androgen and estrogen sex hormone pathways, with distinct roles in each gender.

Progenitor cell derived hepatocytes accrue in chronic hepatitis, possibly related to native hepatocellular dysfunction. In this study, no HPC were seen just around hepatocellular carcinomatous nodules. HPC were found in the desmoplastic areas away from the carcinomatous cells. Also seen forming ductules & cords around the cirrhotic nodules with or without neoplasia related changes. This is similar to the results of Roncalli *et al*. [19] stating that ductular reaction (DR) was commonly seen in the isolated, intranodular portal tracts and in the outer capsule as well.

HPC in this study were observed in and around portal tracts and along the fibrous septa, mostly in small clusters, cords or forming ductules. DR was so evident in cirrhotic cases. No intra-acinar HPC were observed. Guimei *et al*. [15] also observed DR in and around portal tracts and along the fibrous septa with few intra-acinar positive cells.

A statistically significant directly proportionate relationship was detected between HPC & age, portal inflammation, interface hepatitis and lobular inflammation. Clouston *et al*. [21] found a close relationship between HPC & aggregate necroinflammatory activity (by Ishak scoring) and also for the individual inflammatory components of interface hepatitis, lobular inflammation and portal inflammation.

A statistically significant directly proportionate relationship was detected between HPC & Ishak HAI grade & stage. Mild Ishak HAI (grades 0 & 1) showed no HPC. This is in agreement with Guimei *et al*. [15] who mentioned that DR correlated with the grade and stage in hepatitis cases. Also in the study performed by Tsamandas *et al.* [22] HPC-percentages were directly correlated with total HAI score, fibrosis stage and transaminase values.

Concerning hepatic progenitor cells in this study, values were in range (0-18) in non-cirrhotic HCV. Values were in range (18-50) in cirrhotic HCV with minimal cutoff point eighteen cells. This differs from the values obtained by Guimei *et al*. [15] who use CK19 as a marker for HPC. Values were in the range of 0–10 and 0–90 for non-cirrhotic hepatitis and cirrhosis cases, respectively. This may be due to the use of different immunomarkers.

The significant association of HPC expression with the severity of disease and more specifically with the response to treatment implies that HPC development and proliferation may predict prognosis in HCV treatment.

c-kit positive HPC were found in 48/60 studied HCV cases (80%). The twelve c-kit negative cases showed mild grade & stage with no HPC noticed as regards morphology in H&E stain. However, some HPC ductular reaction in H&E stain didn't take c-kit immunostain within the positive cirrhotic cases. Koruk *et al.* [23] said that c-Kit positive oval cells were found in 30/61 (49.1%).

Tsamandas *et al*. [25] mentioned that the number of CK19 positive HPC varied from 22±4 to 89±6 in chronic hepatitis C. The number of GST- πpositive oval cells varied from 21±4 to 75±3 in chronic hepatitis C. These figures were higher than those of HPC stained by c-kit in the current study.

A statistically significant directly proportionate relationship was detected between HPC & METAVIR activity and stage of fibrosis. Minhui *et al.* [26] mentioned that DR significantly correlated with necroinflammatory grade and fibrotic stage in hepatocellular carcinoma. In this study, a statistically significant directly proportional relationship was detected between HCC & HPC with minimal cutoff twenty cells (P value> 0.001). Minhui *et al.* [26] mentioned that a continuing necroinflammatory microenvironment stimulates HPC expansion to form DR and partly to be diverted to malignant direction.

Knight *et al*. [27] mentioned that selective inhibition of HPC growth during preneoplastic injury may prevent or delay the onset of liver cancer. Rats carrying a germ-line mutation in c-kit have an impaired HPC response to liver injury. Tyrosine kinase inhibitors, c-kit inhibitor imatinib mesylate, would suppress HPC growth and, therefore, may exert antitumorigenic effects in the liver. This link between cirrhosis and stem cell proliferation; is multifaceted and complex with interplay of multiple factors that enhance or inhibit the proliferative rate of HPC, which culminates in HCC development [15].

CONCLUSIONS

Hepatic progenitor cells are well correlated with necroinflammatory grades and fibrosis stages. They are also directly proportional to hepatocellular carcinoma with minimal number twenty cells. A close search and reporting of HPC could be useful in predicting the outcome in HCV patients. Clear and precise delineation of markers specific to HPC is highly recommended as there is no consensus about which specific markers best define HPC or HCC stem cells. Further research by the use of multiple HPC markers for precise identification of sensitivity & specificity of c-kit is needed to reveal the debate about the exact origin of these cells or the possibility of transdifferentiation. More studies are needed on transplantation of progenitor cells to regenerate the liver and to evaluate the use of Imitinab to delay the disease progress and complications.

Abbreviations:

A: METAVIR activity, CK: Cytokeratin, DR: Ductular reaction, EMT: Epithelial mesenchymal transition, F: METAVIR fibrosis, HAI: Hepatic activity index, HCC: Hepatocellular carcinoma, HCV: Hepatitis C virus, HPC: Hepatic progenitor cells, HPF: High-power field, IH: Interface hepatitis, LI: Lobular inflammation, N: number, p: probability, PI: Portal inflammation, r: range, SD: standard deviation.

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