ORIGINAL ARTICLE

Effect of MCP-1 and CCR2 Genes Polymorphism on Development of Hepatocellular Carcinoma in HCV- Infected Patients in Sohag Governorate, Egypt

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ABSTRACT

Background: HCC is the most common primary tumor of the liver .It is the fifth common cancer in men and the eighth common in women and is the second leading cause of cancer-related death in the world. The MCP-1 is a chemokine and a potent chemotactic factor for monocytes. MCP-1 expression in tumor cells is significantly linked to the extent of tumor-associated macrophage infiltration. Objectives: to detect the effect of MCP-1 and CCR2 Genes Polymorphism in development of HCC in HCV- related liver cirrhosis patients. Methodology: MCP-1-2518 A/G and CCR2 (V64Ile) genes polymorphism was assessed by real time PCR in 35 HCC patients and 30 HCV related LC. Serum MCP-1 was measured by ELISA. Results: For MCP-1 -2518 A/G gene, HCC patients had a higher frequency of AG and GG genotypes than of AA genotype compared to other groups. For CCR2 (V64Ile), HCC patients had a higher frequency of GA and AA genotypes than GG genotype compared to other groups. Conclusion: There is a significant association between CCR2 (V64Ile) polymorphism, high serum MCP-1 level and HCC development in HCV- related liver cirrhosis patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, representing approximately 90% of primary liver cancer in chronic liver disease and cirrhosis patients [1]. HCC is the most common cancer in men in Egypt, the second most common in women and the most common cancer in both sexes combined. In 2018, HCC incidence increased to 19.7 % [2].

It has been identified that the most important risk factor for HCC development is hepatic cirrhosis. Approximately 80 % HCC develops in cirrhotic livers [3]. Major risk factors for HCC include chronic hepatitis B virus and chronic hepatitis C infection, alcohol, and non-alcoholic fatty liver disease, aflatoxin, genetic factors, obesity, diabetes and smoking [4].

HCV infection is the serious public health problem in Egypt, and is the leading cause of cirrhosis (93%), which is the main risk factor for HCC development [5]. The Egyptian Demographic Health Survey (2008) stated a national sero-prevalnce of HCV (14.7 %) among those aged between 15 and 59 years, with a viraemic prevalence of (9.7 %) at this age, prior to the start of the National Program for the management of chronic HC [6].

Although the relationship between HCV and HCC development is well defined, the pathogenic mechanism of hepato-carcinogenesis is still unclear, including host genetic factors can also contribute to great extent, especially gene polymorphisms of inflammatory cytokines and growth factors receptors and ligands [7].

Monocyte chemo-attractant protein-1 (MCP-1) is a member of a large family of chemokines known to be important soluble mediators of innate immunity and tissue inflammation. MCP-1 has a key role in controlling monocyte/macrophage migration[8]. MCP-1 expression is significantly correlated with the extent of tumor-associated macrophage infiltration in the tumor tissue which is essential to the initiation of tumor arteriogenesis [9].

MCP-1 may promote Th2 development and contributes to cytokine-mediated inflammation. MCP-1 serum and tissue concentrations are influenced by transcriptional activity and by posttranslational regulation [10].

The cell surface receptor that binds MCP-1 (CCR2) is expressed on many types of immune cells, including monocytes / macrophages, basophils, mast cells, T lymphocytes, NK cells and dendritic cells. It was also suggested that the communication between MCP-1 and CCR2 help in the recruitment of activated macrophage into the liver tissue in case of hepatotoxicity and hepatic inflammation [11].

In several forms of toxic liver injury, macrophages recruited by MCP-1 are an essential cofactor for the occurrence of hepatocellular necrosis. These inflammatory cells are the source of hepatocellular damage. It is assumed that liver cell injury renders hepatocytes sensitive to further damage by macrophage products by impairing the ability of hepatocytes to up-regulate normal cellular protection mechanisms [12].

In HCV-infected patients with severe fibrosis, the serum MCP-1 concentrations were significantly higher than in mild liver fibrosis. In general, G allele carriers at -2518 exhibit higher MCP-1 serum levels than those with AA genotype [13].

MCP-1 -2518 A / G and CCR2 (V64Ile) play a key role in HCC development and progression. A polymorphism of promoter region of the G allele at the -2518 A / G MCP-1 position has been detected to increase MCP-1 gene expression. In addition, a G-to-A single-nucleotide polymorphism (SNP) in the CCR2 open-reading frame was established. Also the G / A substitution results in the replacement of valine with isoleucine at amino acid 64 CCR2 (V64Ile) leading to an increase gene expression and a half-life of CCR2 A isoform [14].

In our study, we will focus on the relationship between MCP-1 -2518 A/G and CCR2 (V64Ile) genes polymorphism and development of HCC in different patient categories.

METHODOLOGY

The study is a case control study that was conducted in Medical Microbiology and immunology, Tropical Medicine and Gastroenterology, Biochemistry Departments and Central Research Laboratory, Faculty of Medicine, Sohag University during the period from June 2019 to June 2020.

Seventy patients with liver cirrhosis admitted to the Department of Tropical Medicine and Gastroenterology and twenty-eight age and sex-matched healthy controls were included in the study. Those patients were divided into two groups; Group (1) included thirty- three patients of liver cirrhosis with no evidence of HCC on top of liver cirrhosis. Patients were excluded if they had a benign hepatic focal lesion or any malignant hepatic focal lesion other than HCC. Group (2) included thirty seven patients diagnosed to have HCC on top of liver cirrhosis. Informed oral consent was obtained from patients. The study protocol was approved by the local ethics committee of the faculty.

Complete medical history was taken from the patients and they were subjected to clinical examination, laboratory investigations and abdominal ultrasonography. Triphasic CT scan of the abdomen was done if a hepatic focal lesion was detected by ultrasonography to establish the diagnosis of HCC.

Quantitative estimation of Human MCP-1 concentrations by enzyme linked immunosorbant assay:

Five ml blood in one plane tube centrifuged at 5300 rpm for 20 minutes, serum was collected from the upper part of the tube, was put in 1.5 ml microcentrifuge tube and stored in -20 till ELISA technique done,Using commercially available kit from SinoGene Clon Biotech Co.,Ltd, China. All steps were done following the manufacture's protocol

Genotyping technique for detection of gene polymorphism:

Peripheral blood samples were was collected in ethylene-diamine-tetraacetic acid tubes. Genomic DNA was extracted from the mononuclear cell layer using aQIAamp DNA blood Mini Kit (QIAGEN, Germany) and stored at -20 C. Polymorphism genotyping of MCP-1 -2518 A/ G and CCR2 (V64Ile) SNP was detected by real time PCR. Amplification and detection was carried out by real time PCR STEP 1 **(Thermo fisher, America)**.

Primer sequences:
- For MCP-1-2518 A/G polymorphism; Forward: 5'GGGAACTTCCAAAGCTGCCT3', and Reverse: 5'AGCTTTGCTGGCTGAGTGTT3'.
- For CCR2 (V64Il) polymorphism; Forward: 5'TGCGTGTTTGTGTGTGGTCA 3', and Reverse: 5'AGATGGCCAGGTTGAGCAGGT 3'.

The reaction was conducted in a total of 25 μL reaction volume containing Taq Man universal PCR Master Mix (2X), Noamperase UNG (12.5 μLs) working stock of SNP, primer sequences for CCR2 (V64Il) and (2.5 μL for each forward and reverse sequences), and DNase free water (8.5 μL). The 20 X SNP Genotyping assay was vortexed and centrifuged briefly. The required total volumes of UMM, DNase free water and 20 X SNP genotyping assay were pipette into sterile microcentrifuge tube and inverted several times to mix. 3 μLs of the extracted genomic DNA was added to the tubes in the strip. The tubes we capped and inverted several times to mix.

***The thermal cycling conditions**: (1) Initial 10 minutes at 95 c^o for ampli Taq Gold enzyme activation. (2) Denaturation step 95 c^o for 15 seconds. (3) Annealing at 60 c^o for 1 minute. (4) Primer extension at 72 c^o for 5 minutes. The total number of cycles was 40. The reaction plate was loaded into the thermal cycler, and then the run started. After PCR amplification an end point plate read was performed using an applied Bio systems real time PCR system. The sequence detection system software detected the fluorescence emission which was released during reading the plate and plotted the fluorescence (rn) values based on the signals from each well. The plotted fluorescence signals indicated which alleles are in each sample.
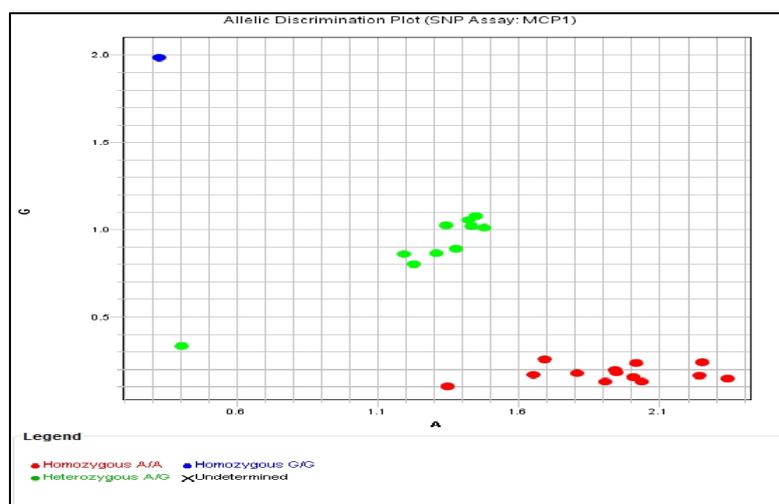
Figure 1: Allelic discrimination of MCP-1-2518 A/ G in HCC patients;The red dots refer to genotype AA, green refer to genotype AG, and blue dots refer to genotype GG.
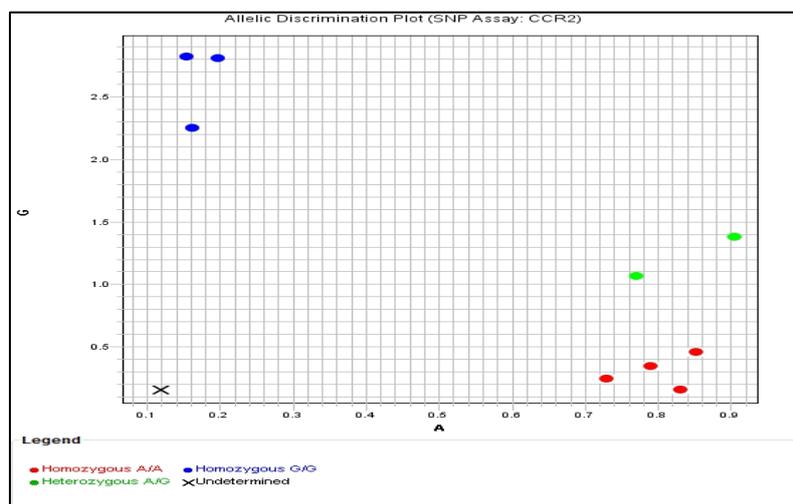
Figure 2: Allelic discrimination of CCR2 (V64Ile) gene among HCC patients: The red dots refer to AA genotype, the green refer to genotype AG, and blue dots refer to genotype GG.

Ethical approval:

The study protocol was approved by local Ethics Committee of scientific Research in Sohag University, Faculty of medicine without any external funding corporation.

Statistical analysis:

Data were analyzed using STATA version 14.2 (Stata Statistical Software: Release 14.2 College Station, TX: StataCorp LP.). Quantitative data were represented as mean, standard deviation, median and range. Student t-test was used to compare means of two groups and ANOVA test to compare means of three groups or more. The odds ratios and their corresponding 95% confidence intervals were calculated to compare the genotype frequencies. P-value< 0.05 was considered significant.

RESULTS

The age, gender, smoking history, history of diabetes or hypertension of the participants were comparable in both study and control groups in table (**1**). Ultrasonography findings of the groups were shown in table (**2**). Laboratory tests including CBC, ALT and AST, PT was significantly higher in the patients, while the albumin level was significantly lower in patients than controls table (**3**). Serum level of MCP-1 was markedly elevated in HCC group than liver cirrhosis group and normal controls table (**4**).
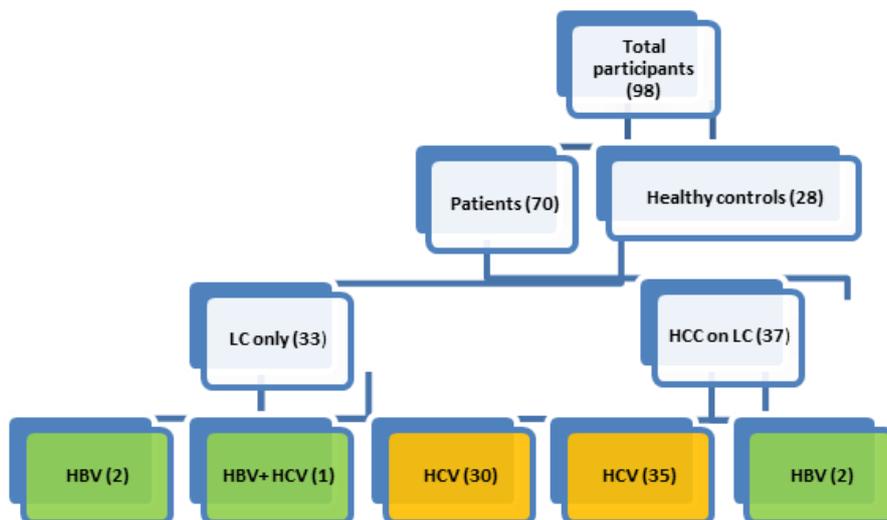
Fig. 3: Flow chart of the patient groups; ■: Excluded patients; ■: Patients included in statistical analysis

Table 1: Socio-demographic characteristics of the participants

Variable	HCC on LC N=35	LC N=30	controls N=28	P-value
Age (years)				
Mean ± SD	62.31±8.09	63±9.99	62.31±8.01	0.95
Median (range)	62 (45:76)	64 (40:86)	60.5 (50:76)	
Sex				
Female (F)	8 (22.86%)	10 (33.33%)	8 (28.57%)	0.64
Male (M)	27 (77.14%)	20 (66.67%)	20 (71.43%)	
Smoking				
No	24 (68.57%)	25 (83.33%)	26 (92.86%)	**<0.0001**
Yes	11 (31.43%)	0 (0%)	0 (0%)	
X-smoker	0 (0%)	5 (16.67%)	2 (7.14%)	
Hypertension	19 (54.29%)	10 (33.33%)	0 (0%)	**<0.0001**
Diabetes mellitus	9 (28.71%)	6 (20.00%)	0 (0%)	**0.02**

Table 2: Ultrasonography findings in HCC and liver cirrhosis patients

Variable	HCC on LC N=35	LC N=30	P-value
Liver size			
Average	12 (34.29%)	15 (50.00%)	0.03
Enlarged	21 (60.00%)	9 (30.00%)	
Reduced	2 (5.71%)	6 (20.00%)	
Portal vein dilatation	18 (51.43%)	3 (10.00%)	<0.0001
Portal vein thrombosis	12 (34.29%)	0 (0%)	<0.0001
Splenomegaly	27 (77.1%)	19(63.3%)	0.01
Ascites	27 (77.14%)	29 (96.67%)	0.02

Table 3: Laboratory investigations of HCC patients and liver cirrhosis patients

Variable	HCC on top of liver cirrhosis N=35	Liver cirrhosis N=30	P value
Hb (g/dl) Mean ± SD	10.18±2.11	11.29±1.93	**0.02**
WBCs (10³/µl) Mean ± SD	9.48±3.68	10.59±4.4	0.56
Platelets (10³/µl) Mean ± SD	92.43±43.46	136.98±47.92	**0.0002**
Fasting Blood glucose (mg/ dl) Mean ± SD	124.69±65.29	118.91±45.48	0.59
Total bilirubin (mg/dl) Mean ±SD	4.9±3.32	6.2±3.09	**0.01**
Serum albumin (g/dl) Mean ±SD	2.26±0.63	2.67±0.67	**0.01**
AST(IU/ l) Mean ± SD	182.86±87.11	48.72±22.18	**0.0001**
ALT(IU/ l) Mean ± SD	111.86±74.41	55.32±24.09	**0.0001**
PT (seconds) Mean ± SD	15.42±3.21	15.01±1.03	1.0
Child classes Child B [N (%)] Child C [N (%)]	13 (37.14%) 22 (62.86%)	9 (30%) 21 (70%)	0.54

Table 4: serum level of MCP-1 in all groups

Serum level of MCP1	HCC N=35	LC N=30	Normal controls N=28	P-value
Mean ± SD(ng/ l)	1027.93±520.25	396.69±90.00	115.01±97.0	
Median (range)	1101.6	399.9	97.5	**0.0001**

The genotypes frequencies in HCC and liver cirrhosis patients and controls group were displayed in **Table (5).** MCP-1 -2518 A/ G gene, HCC patients had AG genotype (57.14%) more frequently than patients with liver cirrhosis (40.00%). Also for GG genotype was higher in HCC patients (25.71%) than patients with liver cirrhosis (16.67%). For AA genotype patients with liver cirrhosis had higher frequency than patients with HCC (43.33% *vs*. 17.14%) with a statistically-significant difference (P-value 0.005).

Regarding CCR2 (V64Ile), HCC patients had more frequent AG genotype (45.71%) compared to patients with liver cirrhosis (26.67%), for genotype AA had higher frequency in HCC patients (42.86%) than patients with liver cirrhosis (20.00%) also with a statistically-significant difference (P-value 0.001).

Table 5: MCP-1 and CCR2 (V64Ile) gene alleles among the studied population

Variable	HCC N=35	Liver cirrhosis N=30	Normal controls N=28	P-value
MCP-1-2518A/ G Gene AA AG GG	6 (17.14%) 20 (57.14%) 9 (25.71%)	13 (43.33%) 12 (40.00%) 5 (16.67%)	18 (64.29%) 8 (28.57%) 2 (7.14%)	**0.005**
MCP-1 -2518A/ G gene alleles A allele G allele	32 (46%) 38 (54%)	38 (64%) 22 (36%)	44 (79%) 12 (21%)	**0.001**
CCR2 (V64Ile) genes GG GA AA	4 (11.43%) 16 (45.71%) 15 (42.86%)	16 (53.33%) 8 (26.67%) 6 (20.00%)	16 (57.14%) 8 (28.57%) 4 (14.29%)	**0.001**
CCR2 (V64Ile) genes alleles G allele A allele	24 (34%) 46 (66%)	40 (67%) 20 (33%)	40 (71%) 16 (29%)	**<0.0001**

Serum level of MCP-1 in HCC group among different genotypes showed that patients with genotypes AG and GG showed higher level MCP-1 than patients with AA genotype as in **table (6)**.

Table (6): Serum level of MCP-1 in HCC group among different genotypes

Variable	AA N=6	AG N=20	GG N=9	P-value
MCP1 (ng/L) **Mean ± SD** **Median (range)**	624.72±399.90 476.4 (214.4-1160)	1185.07±566.07 1245.85 (207.8-1973)	947.53±313.66 1043 (215.7-1299)	0.06

Univariate binary logistic regression analysis revealed that smoking, level of serum MCP-1, AG, GG genotypes of MCP-1-2518 A / G and GA , AA genotypes of CCR2 were the factors significantly associated with HCC development as shown in (table 7). Multivariate binary logistic regression analysis confirmed that smoking, level of MCP-1, Child score C and AA, GA genotypes of CCR2 (V64Ile) gene were independent predictors for development of HCC in patients with HCV-related liver cirrhosis. (Table 8)

Table 7: Univariate, binary logistic regression analysis of predictor variables of HCC in HCV related liver cirrhosis patients.

Variable	Odds ratio (95% CI)	P-value	Adjusted odds ratio (95% CI)	P-value
Age/year <60 60-70 > 70	 1.0 1.38 (0.46:4.11) 0.65 (0.16:2.68)	 0.57 0.56	 1.0 0.98 (0.07:13.72) 0.39 (0.02:8.22)	 0.99 0.55
Sex Female Male	 1.0 1.69 (0.56:5.04)	 0.35	 1.0 0.36 (0.03:3.91)	 0.40
Smoking No Yes/x-smoker	 1.0 2.29 (0.69:7.58)	 0.17	 1.0 21.15 (1.27:352.29)	 **0.03**
Hypertension No Yes	 1.0 2.38 (0.87:6.52)	 0.09	 1.0 2.90 (0.40:20.82)	 0.29
Diabetes No Yes	 1.0 1.38 (0.43:4.47)	 0.59	 1.0 0.61 (0.03:12.96)	 0.75
Child score B C	 1.0 0.73 (0.26:2.05)	 0.55	 1.0 0.04 (0.001:0.94)	 **0.046**
Platelets	0.98 (0.97:0.99)	0.001	0.98 (0.96:1.01)	0.17
Level of MCP-1	1.005 (1.002:1.008)	<0.0001	1.01 (1.00:1.02)	**0.04**
MCP1 Genotypes **AA** **AG** **GG**	 1.0 3.61 (1.08:12.03) 3.9 (0.91:16.79)	 0.04 0.07	 1.0 2.29 (0.15:35.18) 1.64 (0.05:52.62)	 0.55 0.78
CCR2 Genotypes **GG** **GA** **AA**	 1.0 8 (2:31.99) 10 (2.35:42.55)	 0.003 0.002	 1.0 59.50 (0.73:4841.04) 80.23 (1.72:3732)	 **0.001** **0.03**

Table 8: Multivariate logistic regression analysis of predictor variables of HCC in patients with HCV-related LC.

Variable	Adjusted odds ratio (95% CI)	P-value
Smoking		
No	1.0	
Yes/x-smoker	7.42 (1.13-48.72)	**0.04**
Child score		
B	1.0	
C	0.11 (0.01-0.97)	**0.047**
Level of MCP-1	1.01 (1.00-1.01)	**0.001**
CCR2 Genotypes		
GG	1.0	
GA	6.15 (0.65-57.77)	**0.01**
AA	19.50 (1.81-210.61)	**0.001**

DISCUSSION

It has been documented that liver cirrhosis is the most important risk factor for development of HCC. About 80 % of HCC develops in cirrhotic livers[3]. Host genetic factors are also of great importance, can also contribute particularly gene polymorphisms of inflammatory cytokines and growth factors ligands and receptors. Chemokines and their receptors have been detected in most tumors. Chemokines are involved in a broad array of normal host activities that impact cancer; therefore, it is possible that they will be found to have important effects on cancer pathogenesis[15].

In the present study MCP-1 -2518 A/ G and CCR2 (V64Ile) gene polymorphism and serum level of MCP-1 was measured in HCC and HCV related LC patients compared to normal groups. Our study demonstrates that there was male predominance among HCC and liver cirrhosis groups but with no significant difference between the two groups regarding sex, these results agreed with Hung et al.[7], Liu et al.[16] and Mohammed et al.[17] Abd Elaal.[18]. A cross-sectional study reported an association between higher total serum levels of testosterone and risk of advanced hepatic fibrosis and inflammatory activity in males with chronic HCV infections in the USA [19].

Smoking was significantly more common in HCC group compared to liver cirrhosis and control groups. In agreement with our finding, Koh et al. [20] and Abdel-Rahman et al.[21] found that smoking is a risk factor for disease progression and development of HCC in HCV-related chronic liver diseases. This finding disagree with Abd Elaal[18] and Elsayed[22] who found no significant difference between HCV related HCC patients and HCV- related liver cirrhosis regarding smoking.

In our study, HCC patients showed significantly higher mean values of ALT and AST compared to liver cirrhosis patients, this agrees with Lok et al.[23], Mobarak et al.[24], El-Edel et al.[25] and Abd Elaal[18], while, Hung et al.[7] and Mohammed et al.[17] found that HCC patients had significantly higher mean values of ALT only compared to liver cirrhosis patients. This denotes that HCC patients have more hepatic necro-inflammation compared to cirrhotic patients.

Our study demonstrates that both HCC and liver cirrhosis patients had decreased mean platelet count which was significantly lower in HCC patients compared to those with LC , this agrees with Hung et al.[7], El-Edel et al.[25], Mohammed et al.[17] and Abd Elaal[18]. Thrombocytopenia could be attributed to splenomegaly and hypersplenism secondary to portal hypertension or it may due to decreased activity of thrombopoietin (TPO) Afdhal et al.[26]

In our study serum level of MCP-1 protein was significantly higher in HCC patients than liver cirrhosis patients. This due to persistence of HCV infection which is associated with increased hepatic expression of MCP-1, MCP-1 mRNA was expressed in sinusoidal cells Narumi et al.[27] but not in liver-infiltrating lymphocytes, and this agrees with Leroy et al.[28], Lin et al.[29], Sarma et al.[10] and Reichl and Mikulits[30]. Our results agree with Galal and Raafat[31] who reported that serum MCP-1 can be adjuvant biomarker to AFP for detection of HCC.

Our study demonstrates that the majority of patients in liver cirrhosis and HCC groups had advanced liver cell failure with no significant difference between both groups. Advanced liver cell failure is not the main risk factor for development of HCC. HCC can develop in patients with well compensated liver cirrhosis or even without cirrhosis (as in cases of HBV infection and in NAFLD) (Yeh et al.[11]; Yasui et al.[32]; Gao et al.[33], and Abd Elaal[18]).

In the present study, HCC patients had significantly higher frequency of portal vein thrombosis (PVT) compared to liver cirrhosis patients, this may be related to invasion of the portal vein by malignant thrombus[34]. Our results agree with Tarantino et al.[35], Cagin et al.[36] and Abd Elaal[18] who found that the development of PVT was associated with the severity of liver disease and HCC, and it may be a sign of advanced tumor stage.

The present study showed that patients with HCC had a significantly higher frequency of MCP-1-2518 A/G AG, GG genotypes compared to control subjects and liver cirrhosis patients, our results agree with Mansour et al.[12] and Da et al.[37] and don't agree with Nahon et al.[38] and Yeh et al.[11]. Da et al.[37] reported that carriage of GG genotype was associated with increased risk of cancer of digestive system compared to those carrying AA and AG genotypes

Studying SNP of CCR2 (V64Ile) gene in our study revealed that GG genotype is the predominant variant in liver cirrhosis and control group which agree with Mansour et al.[12]. Both GA and AA genotypes were more frequent in HCC than the other 2 genotypes which agree with Yeh et al.[11] and Mansour et al.[12].

Our finding of a lack of significant association between serum level of MCP-1 and MCP-1-2518 A/G genotypes agrees with Nahon et al.[38] in their study on HCV- related liver cirrhosis.

Despite the small number of the recruited participants, which is the main obstacle of the current study due to lack of financial support , the current study demonstrated the role of MCP-1 -2518 A / G and CCR2- (V64Ile) gene polymorphism and serum level of MCP-1 in patients with HCV infection. In the future, it may help to distinguish more the more risky patients for development of HCC in HCV infected patients who may get benefit in adequate follow up and trail of treatment.

CONCLUSION

The current study suggests a significant association between CCR2- (V64Ile) polymorphism (AA, GA genotypes) high serum level of MCP-1 protein and HCC development and disease severity in patients with HCV-related liver cirrhosis. The CCR2 AA, GA genotype may be used as a molecular marker to predict the risk of HCC in patients with HCV-related liver cirrhosis. The serum level of MCP-1 should be taken in consideration as regard patients with liver cirrhosis and HCC. The distribution of MCP-1and CCR2 genetic polymorphisms should be taken in consideration in patients with non-cirrhotic HCC to reveal if there is relationship. Further studies should be done on patients with liver cirrhosis due to other etiologies (e.g. HBV) to evaluate the impact of MCP-1 and CCR2 gene polymorphisms and serum level of MCP1 on the risk of HCC development.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.

- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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