

Correlation between Vitamin D Receptor and Monocyte Chemotactic Protein-1 Polymorphisms and Spontaneous Bacterial Peritonitis in Decompensated Liver Disease

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ABSTRACT

AIM: In this study, we intended to investigate whether MCP-1 and vitamin D receptor (VDR) polymorphisms are associated with increased incidence of SBP in cirrhotic patients. Additionally, we aimed to assess the levels of vitamin D, MCP-1 and other biochemical markers in such patients.

MATERIALS AND METHODS: A prospective case control study was performed on (60) patients with post-hepatitis C virus decompensated liver disease divided into Group I ($n=30$) patients with SBP and Group II ($n=30$) patients with no SBP; and a control group of 30 healthy volunteers. Estimation of serum MCP-1 and vitamin D polymorphisms by real time PCR and serum level of vitamin D by ELISA for patients and control groups; ascitic fluid level of MCP-1 by real time PCR for patients groups; and quantitative gene expression of MCP-1 in serum and ascitic fluid for patients groups was done.

RESULTS: Our study revealed that MCP-1 and VDR polymorphisms heterozygote genotypes AG in MCP-1 and Ff in VDR were mostly linked to SBP patients. Conversely, one

homozygous genotype was linked to cirrhotic GG in MCP-1 and ff in VDR, while the other was linked to the remaining healthy controls AA in MCP-1 and FF in VDR. A and F alleles may be considered protective factors in healthy controls, while G and f alleles may be considered risk factors. Serum vitamin D was significantly lower and MCP-1 (both serum and ascitic fluid) was significantly higher in SBP group in comparison to cirrhotic and control groups.

CONCLUSION: Higher level of MCP-1 could be an early predictor of SBP. Vitamin D deficiency in cirrhotic patients may be among risk factor for SBP.

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Key words: MCP-1 polymorphisms; MCP-1 gene; VDR polymorphisms; Vitamin D; Liver disease; SBP; Ascites; Cirrhosis

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a serious morbidity global liver complication that is defined as a monomicrobial infection of ascitic fluid in the absence of a contagious source of infection^[1]. Consequently, it is a severe and potentially life threatening complication in patients with decompensated liver cirrhosis^[2]. SBP caused by many etiologies due to the alterations of the immune system that are very common in patients with end-stage liver disease and associated with an increased risk of infection and death^[3,4]. Advanced liver disease and low ascitic fluid protein concentration

have been identified as predisposing factors for SBP^[5]. There is evidence that the most powerful predictive factor for SBP is an ascitic fluid total protein level \leq 1gm /dL which reflects a low complement concentration and decreased opsonisation capacity^[6].

Vitamin D, vitamin D related receptor peptide and vitamin D polymorphism are involved in many infectious diseases. Vitamin D insufficiency may lead to increase in incidence of infections in cirrhotic patients' especially spontaneous bacterial peritonitis^[7].

The leukocytes, infections and inflammation are combined with the presence of monocyte chemotactic protein-1 (MCP-1). MCP-1 belongs to the CC chemokine super family and plays a critical role in the recruitment and activation of leukocytes during acute inflammation. Activated monocytes and fibroblasts may generate MCP-1 by lipopolysaccharide (LPS) or cytokine stimulation^[8].

In this study, we intended to investigate whether MCP-1 and vitamin D receptor polymorphisms are associated with increased incidence of SBP in cirrhotic patients. Additionally, we aimed to assess the levels of vitamin D, MCP-1 and other biochemical markers in such patients.

PATIENTS AND METHODS

Study Design: Prospective case control study.

Study Setting: The included patients were collected from Tropical Medicine Department, Ain Shams University Hospital in the period from January 2013 to October 2013. The study protocol was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Sample size: By using statistical methods sixty (60) patients were required to achieve an alpha error of 5% with a test power of 10%. This study included sixty (60) patients with post-hepatitis C virus decompensated liver disease. They were categorized into two groups according to the presence of spontaneous bacterial peritonitis (SBP) as follows:

Group I ($n=30$) included patients with SBP.

Group II ($n=30$) includes patients with no SBP.

A control group of 30 healthy volunteers were included in our study.

Patients in group I were diagnosed to have SBP according to Rimola *et al.*, 2000^[9]. Patients in group II were those with ascites related to decompensated liver disease and had no clinical symptoms or signs indicating SBP. A written informed consent to participate in the study was obtained from all participants before enrollment in the study. Those with evidence of infection at any site other than SBP were excluded. Patients with alcoholic liver cirrhosis, Wilson disease, hemochromatosis, glycogen storage disease, and malignant or tuberculous ascites were excluded from this study. All patients were subjected to history taking and thorough clinical examination. Laboratory investigations including CBC, Liver profile, prothrombin time "PT", Kidney functions, serum MCP-1 and vitamin D polymorphisms by real time PCR and serum level of vitamin D by ELISA for patients and control groups and quantitative gene expression of MCP-1 in serum and ascitic fluid for patients groups. The following parameters were assessed in ascitic fluid samples; Total protein, Serum Ascites Albumin Gradient (SAAG)^[10], Absolute polymorph nuclear leukocyte count "APLC" ascites, and Ascitic fluid level of MCP-1 By real time PCR for patients groups.

Estimation of MCP-1 and vitamin D

MCP-1 polymorphisms

Genomic DNA was prepared from venous blood samples on EDTA

using the Innu PREP blood DNA mini kit (Analytic jena, Germany) following the manufacturer's instructions. The identification of the polymorphism was carried out using PCR, followed by a restriction fragment length polymorphism (RFLP) assay, using a PvuII site, which is introduced by the presence of the G nucleotide. The regulatory region of the MCP-1 gene (from -2746 at -1817) was amplified by polymerase chain-reaction (PCR) using the following specific primer:

Forward: 5'-CCGAGATGTTCCCAGCACAG-3' and

Reverse: 5'-CTGCTTTGCTTGTGCCTCTT-3'^[11].

PCR was performed using buffer 10 \times (10 mM Tris-HCl pH 9, 2.0 mM MgCl₂, 50 mM KCl), 200 μ M dNTPs, 2.5 pmoles of each primer, 5 μ L of DNA, 0.5 U Taq polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and ddH₂O up to a final volume of 40 μ L. The following thermal profiles were run: 95°C for 40 sec, 56°C for 30 sec, and 72°C for 4 min. After a final extension of 10 min at 72°C, 7 μ L of the PCR products were resolved in 2% agarose gels stained with ethidium bromide previous dilution in blue juice buffer to check the expected 930-bp band. After checking, 8 μ L of the PCR products were digested with 10 U of PvuII in 10 \times buffer and H₂O up to a final volume of 20 μ L at 37°C for 2 hr. The resulting products were separated by gel-electrophoresis in 1.5% agarose gels, containing ethidium bromide in a final concentration of 0.5 g/mL. Samples showing only a 930 bp band were assigned as A/A, samples showing two bands of 708 and 222 bp were considered G/G and samples showing three bands at 930, 708 and 222 bp were typed A/G.

Vitamin D receptor (VDR) polymorphisms

DNA was used in the PCR amplification of sequences containing previously described VDR restriction-fragment-length polymorphisms defined by the restriction endonucleases FokI. The primer sequence used in this study was as follows:

Forward: 5'-AGCTGGCCCTGGCACTGA CTCTGCTCT-3' and

Reverse: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'.

The cycling profile involved denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec for 35 cycles. Final extension was continued at 72°C for 5 min. The amplification procedure was carried out in a PCR thermal cycler (Thermo Scientific, Fenland). PCR products were digested overnight with restriction endonuclease in accordance with the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). Digested products were analyzed by electrophoresis in a 2% agarose gel and ethidium bromide staining^[12].

Quantitative assessment of MCP-1 gene expression by Real time PCR

A. RNA extraction from blood and ascitic fluid samples: using SV Total RNA Isolation system (Promega, USA) according to manufactures instructions.

B. cDNA synthesis: 5 μ g of the extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene USA).

C. Real-time quantitative PCR (qPCR) using SYBR Green I: qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets were optimized at the annealing temperature. All cDNA including previously prepared samples, internal control (for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate. The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a

48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of target gene were measured relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the $\Delta\Delta C_t$ method. We used 1 μ M of both primers specific for each target gene. MCP-1 gene was amplified using the following same primer sequence used for MCP-1 polymorphism assessment. GAPDH gene was amplified using the following specific primer:

Forward: 5'CGCTCTCTGCTCCTCTGTT 3' and
Reverse: 5' CCATGGTGTCTGAGCGATGT 3'^[13].

Quantitative assessment of vitamin D serum levels

ELISA kits were supplied by (R&D system Minneapolis, USA) to assess serum levels of vitamin D (ng/mL) in all studied groups. The techniques were done according to manufacturer references.

Statistical analysis

Results were disclosed as means \pm standard deviations. One-way ANOVA and Tukey's multiple comparison post hoc tests were performed. $P < 0.05$ was considered significant.

RESULTS

Baseline demographic and clinical characteristics

The demographic and clinical characteristics of the studied groups are represented in table 1. As outlined in Table 1, all patients were Egyptian Arabs predominantly middle age males. They had decompensated liver disease with or without SBP and the healthy controls were age and sex matched. There was no statistically significant difference between both studied groups of patients regarding the clinical characteristics data.

Genotypes distribution and alleles frequencies of MCP-1 polymorphisms

Figure 1 illustrates MCP-1 polymorphisms genotypes distribution and allele's frequencies in all studied groups. Healthy controls genotypes distribution didn't depart from those on the basis of Hardy-Weinberg equilibrium and AA was dominant by 53.33%. However, cirrhotic patients dominated GG genotype by 66.66% and SBP group dominated AG genotype by 73.33% with a significant difference ($P \leq 0.05$) for both from control and each other. Also, a significant association of the G allele frequency appears for both groups of patients (cirrhotic 76.66% and SBP 53.33%) when compared to healthy volunteers and each other ($P \leq 0.05$). Contrarily, the A allele 73.33% is predominant in healthy controls.

PCR products for MCP-1 gene (930 bp) appeared in figure 2A before cutting with restriction enzyme for different groups and after

Table 1 Baseline demographic and clinical characteristics of studied groups.

	Group I (SBP) (N = 30)	Group II (Cirrhotic) (N = 30)	Group III (Control) (N = 30)
Age (years)	52 (8.8)	48 (12.6)	48 (4.5)
Sex (male)	21 (75%)	17 (56.7%)	42 (75%)
BMI (kg/m ²)	31.2 (7.1)	30.2 (4.8)	31.5 (6.9)
DM (Yes)	7 (23.3%)	9 (30%)	3 (10%)
GIT bleeding (Yes)	6 (20%)	6 (20%)	0
Hepatic encephalopathy (Yes)	11 (36.7%)	10 (33.3%)	0
Duration of liver cirrhosis (years)	5 (5.1)	4 (3.3)	0
Duration of ascites (years)	2 (2.9)	2 (2.88)	0
US ascites grade:			
Moderate	12 (40%)	11 (36.6%)	0
Marked	18 (60%)	19 (63.3%)	0
SBP symptoms (yes)	29 (96.6%)	0	0
Previous attacks of SBP (yes)			
Child Class	7 (23.3%)	0	0
Class B	7 (23.3%)	12 (40%)	0
Class C	23 (76.7%)	18 (60%)	0

Data are means (SD) or numbers (%).

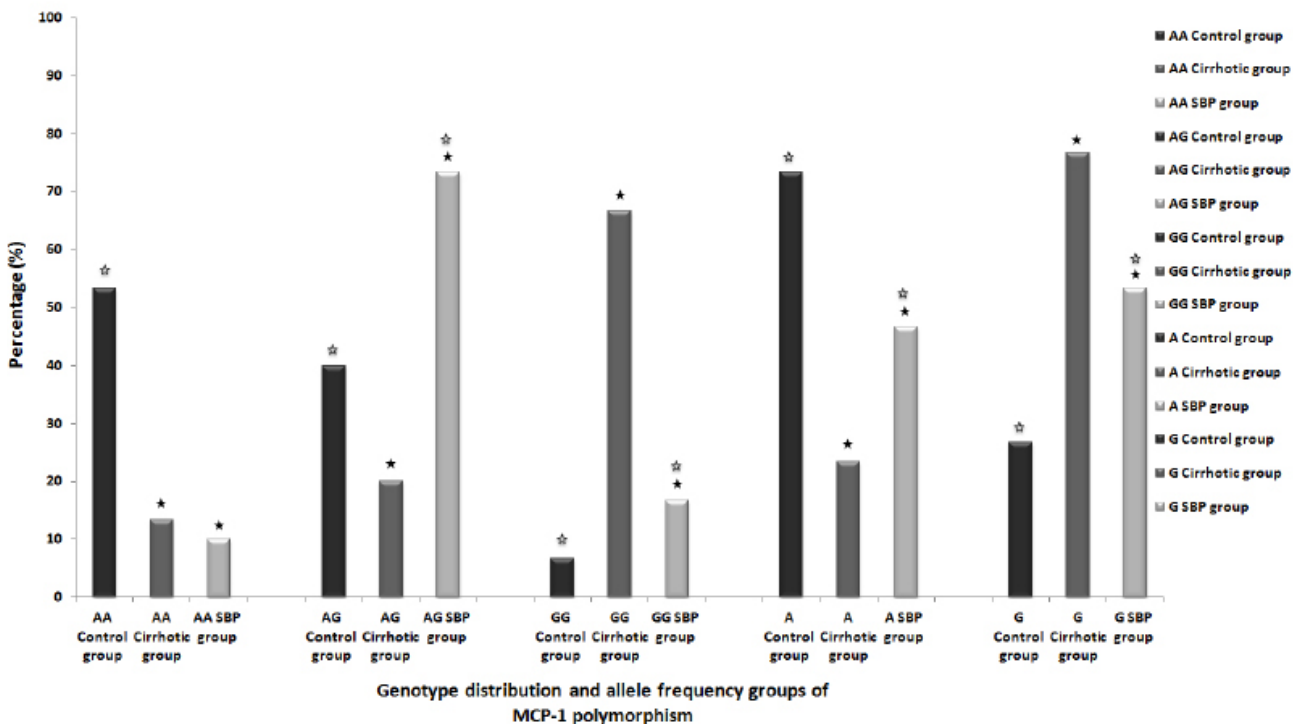


Figure 1 The percentage distribution of genotypes and alleles frequencies of MCP-1 polymorphism versus studied groups (control, cirrhotic and SBP). Data are means \pm SD, (*) p value < 0.05 compared with control group and (☆) p value < 0.05 compared with cirrhotic.

(Figure 2B) cutting with restriction; A/A genotype at (930 bp), A/G genotype (Figure 2C) at (930 bp, 708 bp and 222bp) and G/G genotype at (708 bp and 222bp).

VDR polymorphisms genotypes distribution and allele's frequencies

VDR polymorphisms genotypes distribution and allele's frequencies are shown in figure 3. The healthy controls genotypes distribution dominated FF genotype that was 86.66%. On the other hand, cirrhotic group dominated ff genotype by 56.66% and SBP group dominated Ff genotype by 43.33% with a significant difference ($P \leq 0.05$) for both from control and each other. Association with, a significant connection of the f allele frequency appears for both groups of patients (cirrhotic 68.33% and SBP 61.66%) when compared to healthy volunteers and each other ($P \leq 0.05$). Contrarily, healthy control group is associated

with the F allele by 88.33%.

PCR products restricted with FokI were showed in figure 4. Three bands after treatment with enzyme were showed as FF homozygotes (266 bp), Ff heterozygotes (193 bp), and ff homozygotes (73 bp) according to the restriction pattern (Figure 4).

Vitamin D, MCP-1 and albumin levels:

Vitamin D level is normal as expected in the control healthy group (III) that appeared in figure 5A. Consequently, due to liver disease a significant reduction for both cirrhotic (II) and SBP (I) groups (15 ± 5.4 and 8 ± 3.4 ng/mL; $P \leq 0.05$ when compared to control), respectively.

MCP-1 gene expression was elevated in serum and ascitic fluid samples of both cirrhotic and SBP groups significantly versus serum level of control group with $P \leq 0.05$ as shown in figure 5B.

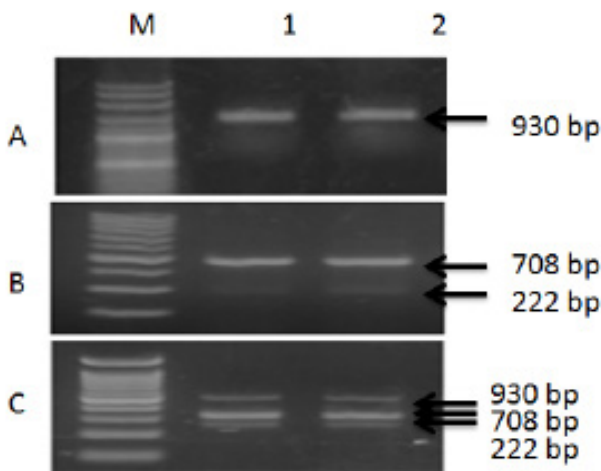


Figure 2 Agarose gel electrophoresis for PCR products for MCP-1 gene: Lane M: DNA ladder (100, 200, 300,1000); Lane 1 & 2 (A): PCR product for A/A genotype (930 bp); Lane 1 & 2 (B): PCR product for G/G genotype (708 bp and 222bp); Lane 1 & 2 (C): PCR product for A/G genotype (930 bp, 708 bp and 222bp).

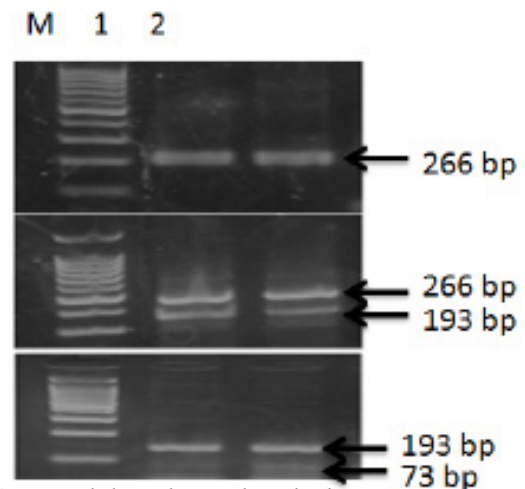


Figure 4 Agarose gel electrophoresis showed FokI restriction patterns of the various genotypes of VDR: Lane M: DNA ladder (100, 200,bp); Lane 1 & 2 (A): 266 bp PCR products (FF genotype); Lane 1 & 2 (B): 266, 193 and 73 bp PCR products (Ff genotype); Lane 1 & 2 (C): 193 and 73 bp PCR products (ff genotype).

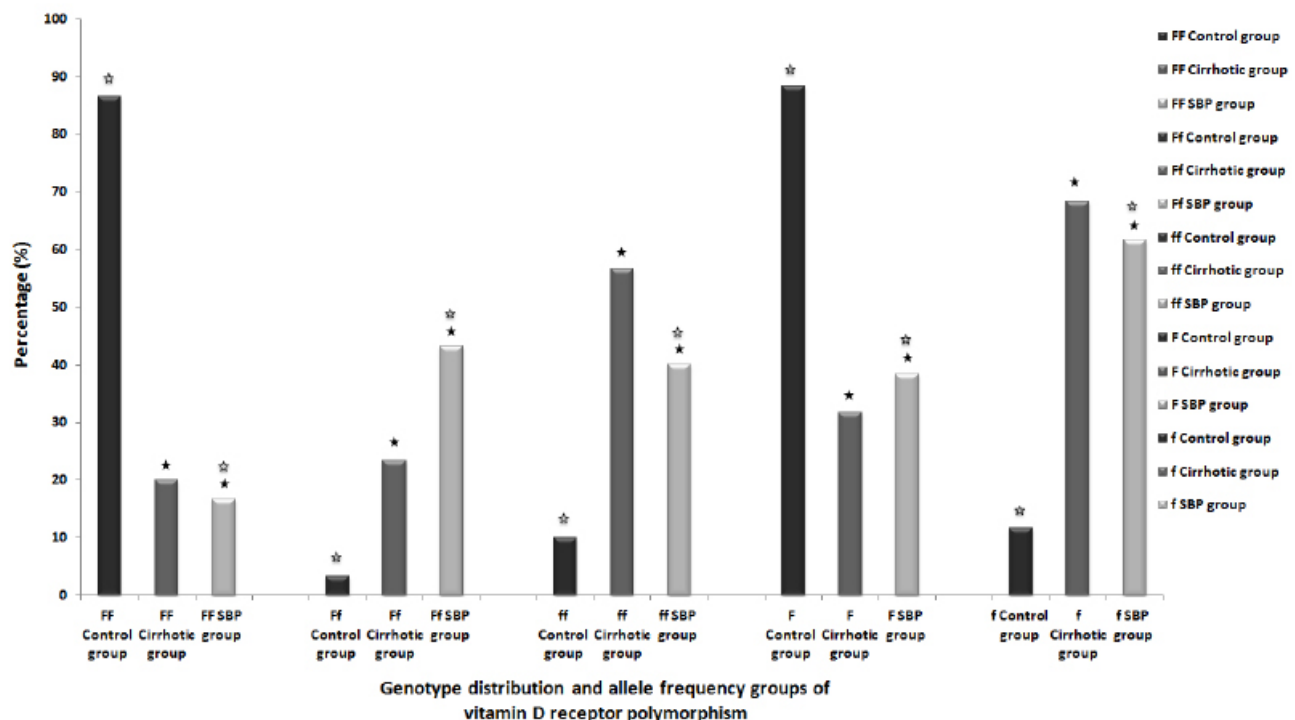


Figure 3 The percentage distribution of genotypes and allele frequencies of vitamin D receptor polymorphism versus studied groups (control, cirrhotic and SBP). Data are means \pm SD, (\star) p value < 0.05 compared with control group and (\star) p value < 0.05 compared with cirrhotic.

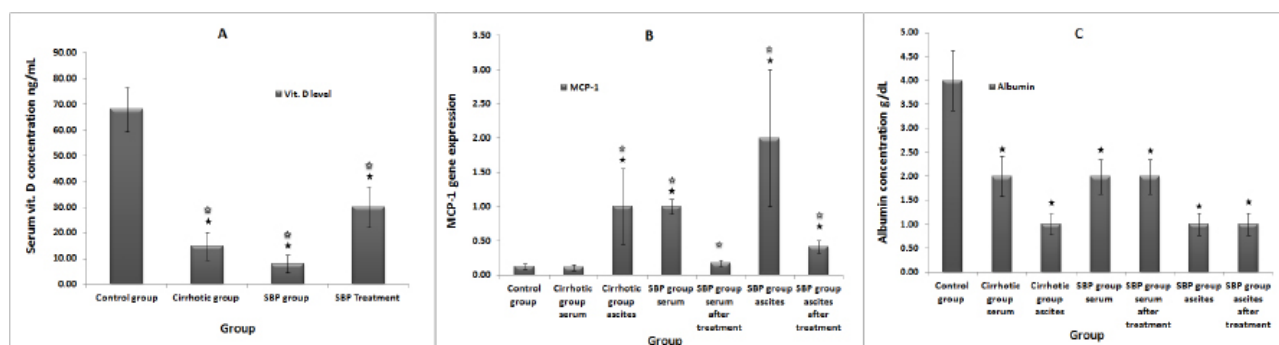


Figure 5 (A) Serum vitamin D concentrations, (B) MCP-1 gene expression, (C) Albumin concentrations changes; serum or ascitic; for studied groups (control, cirrhotic and SBP) before and after vitamin D and antibiotics treatment of SBP. Data are means \pm SD, (★) p value < 0.05 compared with control group and (☆) p value < 0.05 compared in between cirrhotic and SBP groups.

Figure 5C shows a significant ($P \leq 0.05$) reduction in albumin levels in all groups against healthy control.

DISCUSSION

The investigated MCP-1 polymorphisms patients' results clarified the correlation between SBP dominated with AG genotype and cirrhotic patients dominated with GG genotype, while healthy controls dominated AA genotype. Accordingly, the G allele frequency was significantly higher in both SBP and cirrhotic patients as a risk factor in contrarily with healthy controls carrying the A allele as protective factor.

In the same side, VDR polymorphisms examined analysis observation justified the association between SBP dominated with Ff genotype and cirrhotic patients dominated with ff genotype, while healthy controls dominated FF genotype. Consequently, the f allele frequency was significantly higher in both SBP and cirrhotic patients as a risk factor in contrarily with healthy controls carrying the F allele as protective factor.

The two polymorphisms agree in between that; the heterozygote genotypes AG in MCP-1 and Ff in VDR were mostly linked to SBP patients. On the other hand, one homozygous genotype was linked to cirrhotic GG in MCP-1 and ff in VDR, while the other was linked to the remaining healthy controls AA in MCP-1 and FF in VDR. The protective allele factors A and F were present in healthy controls, while G and f alleles were considered as risk factors.

The literature was extensively surveyed; MCP-1 results confirmed with Gäbele *et al.*^[14], who reported that G-allele polymorphism carriers were more frequent with alcohol induced cirrhosis patients than in heavy drinkers without evidence of liver damage. In vitro stimulated monocytes from G-allele carrying subjects produced MCP-1 more than cells from AA homozygous subjects^[15] and were significantly more frequent in HCV patients with more advanced fibrosis and severe inflammation^[16].

To the best of our knowledge, this study was conducted for the first time on SBP cirrhotic patients to study its association with VDR polymorphism. Similar confirmatory results were observed for Hepatitis B, tuberculosis and leprosy patients^[12,17,18]. They all share that VDR polymorphism Ff heterozygote genotype was linked with the examined diseases (SBP in our study).

As expected, vitamin D level decreased in both cirrhotic and SBP patients because of liver disease^[19] and also may be due to VDR polymorphism. Zhang *et al.*^[7] concluded that vitamin D insufficiency was universal among cirrhotic patients with ascites, and the situation was more severe with more serious cirrhosis. Recently, a decreased

vitamin D levels were reported to be associated with increased liver damage and mortality in alcoholic liver diseases. Trépo *et al.*^[20].

In the present study, the mean level of MCP-1 gene expression in serum was significantly higher in patients with SBP than control subjects. A study done by Giron-González *et al.* in 2001^[21] found a non significant increase in the mean value of MCP-1 in serum of patients with SBP than control subjects. They related their results to activated immune and inflammatory reactions in these patients and subsequently, proinflammatory cytokines are elevated.

In our study, there was a significant increase of the mean level of the relative MCP-1 gene expression in both serum and ascitic fluid of SBP rather than cirrhotic ascitic patients without SBP. This agrees with previous researches^[14,15,20] suggesting that MCP-1 plays a pathophysiological role during the development and the course of SBP.

Similarly, Kim *et al.* (2007)^[22] got the same results and explained the role of MCP-1 in SBP. The immune system is stimulated by bacterial invasion. MCP-1 acts as a chemotactic factor for monocytes and macrophages; thus, these cells migrate into the ascitic fluid. These monocytes and macrophages release TNF- α and other cytokines, which in turn induce the expression of adhesion molecules on endothelial cells, thereby mediating a systemic reaction to the infection.

Giron-González *et al.*^[21] found that MCP-1 levels in ascites were significantly higher when compared with their levels in serum, suggesting a chemotactic gradient towards the peritoneal cavity, even in the absence of infection. This chemotactic gradient could be operative in the chemotaxis of monocytes/macrophages and thus might also modify the systemic response to the infection.

In conclusion, MCP-1 and VDR polymorphisms heterozygote genotypes AG in MCP-1 and Ff in VDR were mostly linked to SBP patients. Conversely, one homozygous genotype was linked to cirrhotic GG in MCP-1 and ff in VDR, while the other was linked to the remaining healthy controls AA in MCP-1 and FF in VDR. A and F alleles may be considered protective factors in healthy controls, while G and f alleles may be considered risk factors. Serum vitamin D was significantly lower and MCP-1 (both serum and ascitic fluid) was significantly higher in SBP group in comparison to cirrhotic and control groups. Higher level of MCP-1 could be an early predictor of SBP. Vitamin D deficiency in cirrhotic patients may be among risk factor for SBP.

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CONFLICT OF INTERESTS

The authors declare that they do not have conflict of interests and received no financial support.

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