

Molecular Detection of Monocyte Chemotactic Protein-1 gene Polymorphism in Patients with Spontaneous Bacterial Peritonitis

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ABSTRACT

Backgrounds and Aim: Patients with cirrhosis and ascites show a higher susceptibility to bacterial infections. Monocyte Chemotactic Protein-1 (MCP-1) secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation. We thought to confirm the association of the functional MCP-1 promoter polymorphism (A-2518G) with spontaneous bacterial peritonitis (SBP). **Methods:** 50 patients with post-hepatitis C virus liver cirrhosis and ascites categorized into two groups, group I: 25 patients with SBP, group II: 25 patients free from SBP, in addition a group of 20 healthy volunteers were included. We assessed the MCP-1 gene polymorphism in blood. **Results:** Significant MCP-1 gene polymorphism was detected in group I & II (p-value=0.001 & 0.02 respectively), group I was significantly associated with AG genotype, group II with GG genotype when compared to healthy volunteers (p-value=0.002 & 0.001 respectively), and G allele was significantly higher in both groups (I & II) (p-value 0.005 & 0.001 respectively). **Conclusion:** MCP-1 GG genotype and G allele in HCV infected patients may be associated with more advanced stage of the disease. AG genotype may increase the susceptibility to spontaneous bacterial peritonitis.

Keywords: Genotype, allele, ascites, liver cirrhosis, gene expression.

INTRODUCTION

Patients with cirrhosis and ascites show a higher susceptibility to bacterial infections, mainly because of the inadequate defence mechanisms⁽¹⁾. Factors influencing the development of SBP in patients with liver cirrhosis are poorly understood⁽²⁾. Previous studies have indicated that peritoneal macrophages of cirrhotic patients might contribute to the control of SBP or influence its associated pathology in

human cirrhosis by producing high quantities of angiogenic peptides and nitric oxide. Accordingly, elevated concentrations of proinflammatory cytokines are found in ascetic fluid of patients⁽³⁾. Also Hepatitis C infection is also associated with increased hepatic expression of MCP-1.

MCP-1 acts as a chemotactic factor for monocytes and macrophages; thus, these cells migrate to the ascetic fluid. These monocytes and macrophages release Tumor necrosis factor - γ

(TNF- γ) and other cytokines, which in turn induce the expression of adhesion molecules on endothelial cells, thereby mediating a systemic reaction to the infection. TNF- γ has been shown to be elevated in the ascetic fluid of SBP patients, stimulating the release of interleukin-8 (IL-8), growth-related protein-8 (GRO-8), and MCP-1 by mononuclear cells or endothelial cells. This release propagates the inflammatory reaction. MCP-1 secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation⁽¹⁾.

The aim of this work was to study the association of the functional MCP-1 promoter polymorphism (A-2518G) with SBP.

MATERIALS & METHODS

After being approved by the Institutional Review Boards (IRBs) of Kasr El-Aini hospital, the present study was conducted on 50 patients with post-hepatitis C virus liver cirrhosis and ascites. These patients were enrolled from Hepatology unit at Kasr El-Aini hospital, Cairo –University in the period from February 2012 to September 2012. Patients were categorized into two groups according to the presence of spontaneous bacterial peritonitis (SBP) as follow, group I (n=25) includes patients with SBP as proved by ascitic fluid polymorphonuclear leukocyte (PMN) count ≥ 250 cells/mm³ (18 males and 7 females), group II (n=25) includes patients with no SBP (15 males and 10 females) and an additional control group III, 20 healthy volunteers (15 males and 5 females) with mean age 48.28 \pm 4.56 years were included in our

study, they were recruited from the members of the medical biochemistry department, faculty of medicine. Patients had urine analysis, chest x-ray through clinical examination 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.

After a written Informed consent to participate in the study was obtained from all participants, they were subjected to a detailed medical history assessment, laboratory investigation (CBC, Liver & renal function tests) and detection of MCP-1 gene polymorphism.

Detection of MCP-1 polymorphism:

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

Forward:

5'-CCGAGATGTTCCCAGCACAG-3'
and

Reverse:

5'-CTGCTTTGCTTGTGCTCTT-3'⁽⁴⁾

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⁽⁵⁾.

Statistical Methods:

The results were analysed using the SPSS computer software package version 9.0 (Chicago,IL,USA). Quantitative data were expressed as mean \pm SD. Differences between two groups were compared by Student's T test. Genotype and allele frequencies were reported with their group percentages and the difference between groups were determined by chi square test. Correlations between data were performed using Pearson & Spearman correlation tests as required. Differences were considered significant at $p < 0.05$.

RESULTS

The demographic and clinical data of the studied groups are represented in table (1). Patients of both groups and the healthy controls were age and sex matched. There was no statistically significant difference between both studied groups of patients regarding the studied laboratory data.

MCP-1 polymorphism in all studied groups is illustrated in table (2), our results showed that the genotype frequencies in the healthy controls didn't depart from those expected on the basis of Hardy-Weinberg equilibrium (p -value=0.76), however, in cirrhotic patients without SBP (group II) and those with SBP (group I), the observed and expected frequencies were significantly different (P -value= 0.02 & 0.001 respectively). When compared to normal healthy volunteers a significant association of the GG genotype was reported with cirrhotic patients with no SBP (group II) (p -value=0.00), while a significant association of the AG genotype was reported with cirrhotic patients with SBP (group I) (p -value=0.002). Moreover on comparing both groups of patients with each other, a significant higher frequency of the GG genotype was reported with cirrhotic patients with no SBP (group II) while a significant higher frequency of the AG genotype was reported with cirrhotic patients with SBP (group I) with p -value=0.00. Also there was a significant association of the G allele and both groups of patients (I & II) when compared to healthy volunteers (p -value = 0.005 & 0.00 respectively), while when comparing both groups of patients with each other it was revealed

that the G allele represents 74% in those with no SBP (group II) vs. 54% in those with SBP (group I), while the A allele represents 26% in those with

no SBP (group II) vs. 46% in those with SBP (group I) and this difference is statistically significant with p-value=0.037.

Table (1): Demographic , clinical and laboratory data of the studied groups.

	Healthy volunteers (n=20)	Group I (SBP) (n=25)	Group II (Cirrhotic) (n=25)
Age (years)	48.28±4.56	51.24±9.3	47.08 ±12.9
Sex (Male)	15 (75%)	18 (72%)	15 (60%)
DM (Yes)	0	5 (20%)	8 (32%)
GIT bleeding (Yes)	0	5 (20%)	5 (20%)
Hepatic encephalopathy (Yes)	0	9 (36%)	7 (28%)
Duration of Liver Cirrhosis (years)	0	4.46 ± 5	4.04±3.26
Duration of Ascites (years)	0	1 ± 2.01	1.71±1.54
Hemoglobin(g/dl)	12.6±1.6	10.27 ±1.95 (a)*	9.5±2.22(a)*
Platelets (10 ³ /μL)	158.4± 12.8	146.6±90.2	118.6 ±35.9(a)*
TLC (10 ³ /μL)	6.3 ±0.97	5.82 ±3.05	6.7±2.58
Serum albumin(g/dL)	4.34±0.62	2.26±0.39(a)*	2.3 ± 0.46(a)*
Total bilirubin (mg/dL)	1.036 ± 0.064	5.42±8.7(a)*	2.69 ±2.65 (a)*
Direct bilirubin (mg/dL)	0.176 ± 0.078	3.07±5.3(a)*	1.44±1.58(a)*
Urea (mg/dL)	17.4 ±3.3	59.45±26.6(a)*	42.8±24.49(a)*
Creatinine (mg/dl)	0.86 ± 0.208	1.96±1.79 (a)*	1.5±0.95 (a)*
AST (IU/L)	47.96 ± 7.7	69.86±38.03(a)	85.8±52.99(a)*
ALT(IU/L)	23.79 ± 7.5	39.7 ±16.03(a)*	40.17±24.76(a)
ALP(IU/L)	95.5 ±19.8	167.9±69.49 (a)*	101.4±39.67
INR	0.996 ± 0.13	1.84± 0.59(a)*	1.66±0.40(a)*
PMN count in Ascites (cells/mm ³)	--	1194.6±1187.6(b)*	110.3±60.89
Serum –Ascites Albumin gradient (SAAG) (g/dL)	--	1.34±0.107(b)*	1.67±0.32

Letter (a) denotes significant difference between control group and each of group I and II at p value < 0.05. Letter (b) denotes significant difference between group I and II at p value < 0.05; while (*) denotes high significance at p value <0.01. Results are expressed as mean ± SD or frequency and percent as required.

Table (2): MCP-1 genotyping in the studied groups

	Genotypes			Alleles	
	GG Count (%)	AG Count (%)	AA Count (%)	A Count (%)	G Count (%)
Healthy volunteers	1(5%)	8 (40%)	11 (55%)	30(75.0%)	10 (25.0%)
Group I (SBP)	4(16.0%)	19(76.0%)(b)* (a)	2(8.0%) (a)*	23(46.0%)(a)*(b)	27(54.0%) (a)*
Group II (Cirrhotic)	16(64%)	5(20%)(b)* (a)*	4(16%)(a)*	13(26.0%)(a)*	37(74.0%)(a)* (b)

Letter (a) denotes significant difference between control group and each of group I and II at p value < 0.05. Letter (b) denotes significant difference between group I and II at p value < 0.05 ; while (*) denotes high significance at p value <0.01.

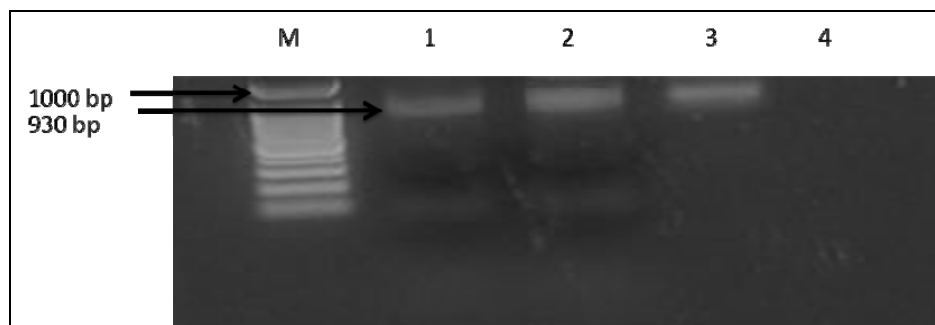


Figure (1): Agarose gel electrophoresis for PCR products for MCP-1 gene (930 bp) before cutting with restriction enzyme.

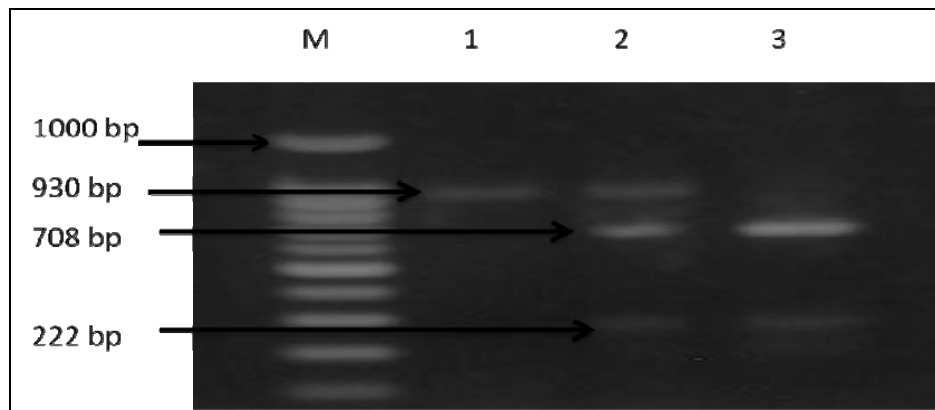


Figure (2): Agarose gel electrophoresis for PCR products for MCP-1 gene (930 bp) after cutting with restriction enzyme.

DISCUSSION

SBP is the most frequent and life-threatening infection in patients with liver cirrhosis⁽⁶⁾. Because inflammatory and immune reactions are altered by hepatic cirrhosis, the efficacy of innate reactions is limited. In these patients, SBP bacterial protein is recognized, and proinflammatory cytokines are released to the blood and ascites⁽⁷⁾.

Monocyte chemoattractant protein-1 (MCP-1) is a cytokine involved in the chemotaxis of monocytes/macrophages and activated lymphocytes during infections⁽⁸⁾.

Interestingly a significant MCP-1 genotype polymorphism was observed in cirrhotic patients with and without SBP in our study, which was undetectable in the healthy Egyptian volunteers, further analysis reported that cirrhotic patients with no SBP were significantly associated with GG genotype, while those with SBP were significantly associated with AG genotype. Also it was reported that the G allele frequency was significantly higher in both the cirrhotic patients with and without SBP than the healthy volunteers as well as being higher in the cirrhotic patients with no SBP than those with SBP. This agrees with Gäbele et al⁽⁹⁾, who reported that carriers of the G-allele of the MCP-1 polymorphism were more frequent in patients with alcohol induced cirrhosis than in heavy drinkers without evidence of liver damage (controls). In vitro stimulated monocytes from individuals carrying a G-allele at -2518 produced more MCP-1 than cells from A/A homozygous subjects⁽¹⁰⁾.

Carriers of the G allele were significantly more frequent in HCV patients with more advanced fibrosis and severe inflammation.⁽¹¹⁾

MCP-1 acts as a chemotactic factor for monocytes and macrophages; thus, these cells migrate to the ascitic fluid. These monocytes and macrophages release TNF- α and other cytokines, which in turn induces the expression of adhesion molecules on endothelial cells, thereby mediating a systemic reaction to the infection⁽¹²⁾ suggesting that this potent chemokine plays a pathophysiological role during the development and the course of SBP. Also in our study the SBP patients showed a significant increase in the mean level of ascitic polymorphonuclear neutrophils (PMN) count than those of cirrhotic patients with no SBP, moreover a significant positive correlation was detected between blood MCP-1 and duration of liver cirrhosis in SBP patients.

Our research study reported that mean level of serum ascites albumin gradient (SAAG) showed a significantly higher level in cirrhotic patients than in SBP patients, this is in agreement with the results of Vedat et al.⁽¹³⁾ where it was shown that the serum ascites albumin gradient (SAAG) was higher in cirrhotic patients than SBP patients but this difference was not statistically significant.

Conclusion:

Inheritance of MCP-1 GG genotype and MCP-1 G allele may be attributed in HCV infected patients to more progressive disease course while AG genotype may be a risk factor to spontaneous bacterial peritonitis in patients with decompensated post

hepatitis C cirrhosis. MCP-1 expression in blood, may be related to the development and the course of SBP.

Recommendation:

Further randomized controlled trials with greater sample size are recommended.

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MCP-1 الكشف الجزيئي عن تعدد الأشكال الجيني للبروتين في مرضى التهاب الصفاق العفوي الجرثومي

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يعد المرضى الذين يعانون من تليف الكبد والاستسقاء أعلى قابلية للالتهابات البكتيرية . يرتفع مستوى الجيني لبروتين الانجذاب الكيميائي وحيد النواة MCP-1 أثناء التهاب الكبد المزمن ويرتبط مع شدة الالتهاب الكبدي.

و قد هدف هذا العمل الي دراسة الارتباط بين تعدد الأشكال الجيني لبروتين الانجذاب الكيميائي وحيد النواة و مرض التهاب الصفاق الجرثومي العفوي.

وقد شملت الدراسة الحالية خمسين مريضا يعانون من التليف الكبدي الناتج من الألتهاب الكبدي C و الاستسقاء و قد تم تقسيمهم الي مجموعتين المجموعة الاولى : شملت ٢٥ مريضا يعانون من التهاب الصفاق الجرثومي العفوي المجموعة الثانيه شملت ٢٥ مريضا بدون التهاب الصفاق الجرثومي العفوي بالإضافة الي مجموعة تشمل ٢٠ شخصا صحيا و قد كانت نتائج هذه الدراسة علي النحو التالي :

- ارتفاع نسبة النمط الجيني (MCP-1 AG) مع المجموعة الاولى و ارتفاع نسبة النمط الجيني (MCP-1 GG) مع المجموعة الثانيه.
 - ارتفاع نسبة النمط الجيني (MCP-1G) في كل من المجموعتين الاولى و الثانيه.
- و بناء علي النتائج السابقة يمكننا أن نستنتج وجود ارتباط قوي بين النمط الجيني (MCP-1 GG) في مرضى التليف الكبدي المضاعف لالتهاب الكبد الفيروسي C والمرحلة المتقدمة من المرض . ان النمط الجيني (MCP-1 AG) يلعب دورا خطيرا في امكانية حدوث التهاب الصفاق الجرثومي العفوي لدي مرضى التليف الكبدي المضاعف لالتهاب الكبد الفيروسي C .