Polymorphism of the Monocyte Chemoattractant Protein 1 Gene in Children with Lupus Nephritis

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Abstract

Background: Lupus nephritis is a major contributor to morbidity and mortality in patients with systemic lupus erythematosus (SLE). Biopsy-proven lupus nephritis (LN) occur in around 80% of all cases of childhood-onset SLE. The etiology of SLE remains unknown. However, there is evidence that both genetic and environmental factors play a role in disease development.

Urinary biomarkers are easily obtained and they specifically represent local renal inflammatory activity. Among the potential urinary biomarkers is Monocyte chemoattractant Protein-1 (MCP-1) which is a chemokine (CC) that is mainly expressed by activated monocytes/macrophages leucocytes and other mediators into sites of inflammation. There is evidence that polymorphisms in the genes of inflammatory mediators may predispose to the development of LN.

Aim of the Work: Our study aimed to examine role of a functional MCP-1 polymorphism in children with SLE and LN and to assess the use of urinary MCP-1 as a marker of disease activity in LN.

Subjects and Methods: This study was conducted on 50 subjects, they included 40 SLE patients (>4 American college of Rheumatology criteria for SLE), 20 with and 20 without LN) and 10 controls.

DNA were obtained and MCP-1 genomic variants were detected by polymerase chain reaction (PCR) followed by Restriction Enzyme Fragment Length Polymorphism (REFLP).

Urinary MCP-1 levels were measured by Enzyme-Liked Immuno Sorbent Assay (ELISA).

Results: The A/A genotype was more common in control (80%) than in SLE patients (52.5%), where as both A/G and G/G genotype were more frequent in SLE patients than in control (p=0.215 for all). The A/A genotype was observed in only 45% of the patients with LN compared with 60% of those without LN. However, these differences did not reach a statistically significant level (p>0.05).

Urinary levels of MCP-1 were significantly higher in patients with LN (p=0.000)

Conclusion: These results may suggest a possible role of genetic polymorphism of MCP-1 in the development of SLE and subsequently LN in children which still need further investigations. Moreover, urinary levels of MCP-1 may be a useful marker for the detection and management of LN.

Key Words: Genetic polymorphism – Lupus nephritis – MCP-1 (monocyte chemoattractant protein-1).

Introduction

LUPUS nephritis (LN) is a major contributor to morbidity and mortality in patients with systemic lupus erythematosus (SLE) [1]. Biopsy-proven lupus nephritis occurs in around 80% of all cases of childhood-onset SLE [2].

There is evidence that both genetic and environmental factors play a role in disease development [3]. While there is emerging knowledge about the regulatory elements of the inflammatory response, less is known about genetic factors that may predispose to development of LN [4].

Urinary biomarkers are easily obtained and probably are best at reflecting the current renal status, as they specifically represent local inflammatory activity. Among the potential urinary biomarkers are TWEAK, Lipocalin-2, and MCP-1 [5].

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine (CC) that is mainly expressed by activated monocytes/macrophages leucocytes and other mediators into sites of inflammation [6,7]. MCP-1 is implicated in the activation of inflammatory cells [8,9] and has been suggested to affect the progression of lupus nephritis (LN) [10]. MCP-1 is consistently found at high levels in the urine of patients with active LN [11,12].

The polymorphism in the MCP-1 gene is suggested to be associated with nephritis in SLE through modulating MCP-1 expression [10]. A 930
bp fragment including the distal regulatory segment of the MCP-1-5' flanking region (nucleotides-2764 to 1817) was analyzed. The product was digested with Pvu-restriction enzyme. Samples with a single 930-bp band were identified as A/A, samples with 3 bands of 930, 708 and 222 bp were typed as A/G and those with 2 bands of 708,222 bp were typed as G/G [13].

Aim of work: To investigate the presence of polymorphism in the gene regulatory region of MCP-1 in SLE children with nephritis, as well as measuring the urinary MCP-1 level to detect their significance in predicting the occurrence of LN, and to relate their variability to the variation in the severity of this disease, in the hope of finding better lines for diagnosing and treating SLE in children.

Subjects and Methods

This study was conducted on 40 SLE patients, chosen from the Rheumatology and Rehabilitation Department and out-patient clinic, in Children Hospital, Cairo University, and fulfilling at least 4 of the American college of Rheumatology criteria for diagnosis of SLE [14] and with age of onset <16 years. Patients were diagnosed to have lupus nephritis (LN) when having persistent urinary sediment (RBC’s >5/HPF or WBC’s >5/HPF in >2 occasions in the absence of infection), persistent proteinuria >0.5g/dl or elevated kidney functions. Renal biopsy was done to these patients and was classified according to the WHO classification [15]. A control group of 10 normal, healthy, sex and age-matched subjects was taken. The consent of all study subjects was obtained. Patients were also subjected to the following laboratory investigations:

- Complete haemogram, using the Counter "Cell Dyne-3500" Abbot Diagnostics USA. Kidney function tests in the form of: Serum urea and creatinine, urine creatinine and protein (were assayed by the Dimension autoanalyzer: From Dade Behring). Complete urine analysis, serum complement levels (C3 and C4), antinuclear antibodies (ANA), anti-dsDNA antibodies, ANA antibodies, anti-dsDNA antibodies were detected by indirect immunofluorescence technique.
- MCP-1 in urine by ELISA (quantitative sandwich technique) using Quantikine Human MCP-1/CCL-2 kit, catalog No DCP00 (from R and D system, Europe, 19 Barton Lane, Abridgin, UK). All urine samples were centrifuged at 200g for 5 minutes to remove cells and precipitates, Urine samples were stored at-8ºC and thawed just before they were analyzed. Urine samples were diluted in PBS, pH 7.0, and HEPES in order to obtain pH stability for all samples. Urine was diluted 1:2. Urinary MCP-1 levels were corrected for urine volume by measurement of urinary creatinine, and values were expressed as the ratio of MCP-1 to creatinine [1].
- Genomic DNA extraction from peripheral leukocytes obtained from EDTA-treated whole blood, using the Purescript DNA isolation kit (Gentra Systems, Minneapolis, MN).
- Genotype screening of MCP-1 regulatory region at position-2518, employing Polymerase chain reaction (PCR), followed by Restriction fragment length polymorphism (RFLP)using the method described by Rovin, et al. (1999) [13]. The PCR master mix kit was provided by Clinilab (Catalog number 201203) and consists of: QIAGEN PCR buffer (contain Tris. Cl, KCl, (NH4) 2SO4, 15 mM MgCl2, PH 8.7 (20ºC), 25mM MgCl2 solution, d NTP Mix (1 0mM each of d ATP, d CTP, d GTP, d TTP), RNase-free water and Taq DNA Polymerase. Primers for MCP-1 distal regulatory region (nucleotides-2746 to-1817) were provided by The Midland Certified Reagent Company Inc. Of Midland, Texas (Lot Number 242709-00521).

Forward primer sequence: 5’-CCGAGATGT-TCGCCACGAC-3’
Reverse primer sequence: 5’-CTGCTTTGCT-TGTGCTCCTT-3’

DNA was amplified by cycling at 94ºC for 3 minutes and then at 94ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 1.5 minutes for 35 cycles, followed by 10 minutes at 72ºC. The product was digested with Pvu II restriction enzyme (New England Biolabs, Beverly, MA) for 16 hours at 37ºC. Detection of digestion products was carried out by agarose gel electrophoresis followed by staining with the fluorescent dye ethidium bromide. The gel was photographed with a Polaroid-type-667-film in a camera fitted with a red-orange filter (Polaroid DS-34). Samples with a single 930-bp band were identified as A/A, samples with 3 bands of 930, 708, and 222bp were typed as A/G, and those with 2 bands of 708 and 222bp were typed as G/G [13].

Statistical analysis:

The results were analyzed using the SPSS computer software package, version 13 (Chicago, USA). Quantitative data were presented as mean ±SD for normally distributed data and as medians and percentiles for skewed data. Qualitative data were presented in the form of frequencies and percentiles. Differences among groups were computed using one-way analysis of variance (ANOVA) with post-
The results of this study are summarized in the following tables and figures:

The SLE with LN included 3 cases of active LN their genotypes were:

2 cases G/G (66.65%) and 1 case A/A (33.35%).

Table (2): Statistical analysis of MCP-1 genotypes in patients and controls.

<table>
<thead>
<tr>
<th>MCP-1 genotype</th>
<th>Patients (n=40)</th>
<th>Controls (n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA genotype</td>
<td>21 (52.5%)</td>
<td>8 (80%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AG genotype</td>
<td>7 (17.5%)</td>
<td>0 (0%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>GG genotype</td>
<td>12 (30%)</td>
<td>2 (20%)</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Table (4): Statistical analysis of allele frequency of MCP-1 in patients and controls.

<table>
<thead>
<tr>
<th>Presence of allele:</th>
<th>SLE (n=40)</th>
<th>Controls (n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28 (59.6%)</td>
<td>8 (80%)</td>
<td>0.224</td>
</tr>
<tr>
<td>G</td>
<td>19 (40%)</td>
<td>2 (20%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency:</th>
<th>SLE (n=40)</th>
<th>Controls (n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>49 (61.2%)</td>
<td>16 (80%)</td>
<td>0.116</td>
</tr>
<tr>
<td>G</td>
<td>31 (38.8%)</td>
<td>4 (20%)</td>
<td></td>
</tr>
</tbody>
</table>

Table (6): Distribution of classes of lupus nephritis in the MCP-1 genotypes.

<table>
<thead>
<tr>
<th>Classes of LN by renal biopsy</th>
<th>MCP-1 genotype by PCR and RFLP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No LN</td>
<td>AA (n=21) AG (n=7) GG (n=12)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Class I</td>
<td>0 (0%) 0 (0%) 0 (0%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Class II</td>
<td>4 (19.0%) 2 (28.6%) 2 (16.7%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Class III</td>
<td>6 (28.6%) 0 (0%) 0 (0%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Class IV</td>
<td>2 (9.5%) 0 (0%) 4 (33.3%)</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Lane 1: Shows the DNA molecular weight marker. Lane 2: Shows AA genotype. Lane 5: Shows GG genotype. Lane 9: Shows AG genotype.

Fig. (1): Genotyping of MCP-1 gene regulatory region at -2518.
Table (7): Clinical characteristics of patients with SLE according to their genotype.

<table>
<thead>
<tr>
<th></th>
<th>AA (n=21)</th>
<th>AG (n=7)</th>
<th>GG (n=12)</th>
<th>Total (n=40)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemia</td>
<td>12 (7.14%)</td>
<td>4 (57.14%)</td>
<td>4 (33.33%)</td>
<td>20 (50%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>1 (4.71%)</td>
<td>0 (0%)</td>
<td>2 (16.7%)</td>
<td>3 (7.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>1 (4.71%)</td>
<td>0 (0%)</td>
<td>2 (16.7%)</td>
<td>3 (7.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Serum urea:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>15 (71.4%)</td>
<td>5 (71.4%)</td>
<td>9 (75%)</td>
<td>29 (72.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Elevated</td>
<td>6 (28.6%)</td>
<td>2 (28.6%)</td>
<td>3 (25%)</td>
<td>11 (27.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Serum creatinine:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>20 (95.2%)</td>
<td>7 (100%)</td>
<td>12 (100%)</td>
<td>39 (97.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Elevated</td>
<td>1 (4.8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (2.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>13 (61.9%)</td>
<td>4 (57.1%)</td>
<td>4 (33.3%)</td>
<td>21 (52.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Casts and cells</td>
<td>13 (61.9%)</td>
<td>2 (28.5%)</td>
<td>6 (50%)</td>
<td>21 (52.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>ANA:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18 (85.7%)</td>
<td>7 (100%)</td>
<td>12 (100%)</td>
<td>37 (92.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Positive</td>
<td>3 (14.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (7.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Anti-dsDNA:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15 (71.4%)</td>
<td>5 (71.4%)</td>
<td>10 (83.3%)</td>
<td>30 (75%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Positive</td>
<td>6 (28.6%)</td>
<td>2 (28.6%)</td>
<td>2 (16.7%)</td>
<td>10 (25%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Serum C3 and C4:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>12 (57.1%)</td>
<td>2 (28.6%)</td>
<td>6 (50%)</td>
<td>20 (50%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Normal</td>
<td>9 (42.9%)</td>
<td>5 (71.4%)</td>
<td>6 (50%)</td>
<td>20 (50%)</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>
Urinary MCP-1 levels:

Urinary MCP-1/creat levels were significantly higher in SLE patients 4.6 (1.3-15.4) compared with controls 1.4 (1.0-2.1) \((p=0.001)\) (Fig. 5-A).

SLE patients with LN had significantly higher MCP-1 levels 6.0 (1.8-21.3) than did SLE patients without LN 2.4 (1.1-4.4) \((p=0.02)\) (Fig. 5-B & Table 8).

Moreover, urinary MCP-1 was markedly elevated in SLE patients with active LN (median 28.4) but they were only 3 cases.

There was a negative correlation between urinary MCP-1 levels and levels of urinary creatinine \((p=0.004)\) and a positive correlation between urinary MCP-1 levels and serum creatinine \((p=0.04)\) and proteinuria \((p=0.000)\).

Discussion

In this study we have found that the prevalence of A/A genotype was more common in control (80%) than in patients (52.5%) while A/G & G/G genotypes were more common in patients (17.5% & 30% respectively) than in control (0% & 20% respectively) but these differences did not reach a significant statistical level (Table 2 & Fig. 2-A,B).

The frequency of A allele in controls (80%) was more common than in patients (61.2%) while the frequency of G allele in patients (38.8%) was more common than in controls (20%) but this difference did not reach statistically significant difference \((p=0.116)\) (Table 4 & Fig. 3).

These results were similar to the results of Marco, et al. [1] who found that A/A genotype was more common in controls than in SLE patients \((p=0.0002)\), where as both the A/G \((p=0.009)\) and G/G \((p=0.0212)\) genotypes were more frequent in SLE patients. In our study we could not find significant difference and this may be due to the lower number of subjects compared with the study of Marco, et al.

In accordance to these results, Rovin, et al. [13] previously reported that in Caucasians and African Americans the G to A allele ratios were 29 to 71% and 22 to 78%, respectively These authors also found a higher frequency of the G allele (47% compared to 53% for the A allele) in Asian subjects.

However, Hwang, et al. in [16] found that the G- vs. A-allele profile in patients with SLE (61 % & 39%) was similar to that in healthy Koreans (65% & 65%), and does not appear to contribute to elevated MCP-1 production in patients.

Another study on Japanese subjects also demonstrated that the ratio of G to A alleles was 65 to
35% [17], indicating that the G allele is indeed the dominant type in Asian populations.

Kim, et al. [10] found the frequencies of MCP-1 polymorphism were similar in SLE patients and control. These results were in agreement with Sanchez, et al. [18] who concluded that no significant difference was found in the allele and genotype frequencies between SLE patients and controls.

In this study we found that in SLE without LN the A/A genotype was more frequent (60%) than in SLE with LN (45%) while the A/G & G/G genotypes were more frequent in SLE with LN (25% & 30%) than in SLE without LN (10% & 30%) (Table 3 & Fig. 4). We did not find a statistically significant association between patients with LN. and patients without LN. regarding MCP-1 genotypes.

The frequency of A allele in patients without LN. (65%) was more common than in patients with LN. (57.5%) while frequency of G allele in patients with LN. (42.5%) was more common than in patients without LN. (35%) (Table 5). However, this difference did not reach a statistical significant level.

Similar to our results Marco, et al. [1] found that patients with LN (n=49) more commonly had the A/G (p=0.0196, OR 2.4) and G/G (p=0.0147, OR 4.5) genotypes compared with those without LN (n=85). They also found that 58% of patients without LN were genotyped as A/A, whereas only 23% of patients with LN had this genotype (p=0.0001). They found similar results when either the presence or the frequency of the A and G alleles was calculated. Presence of the G allele occurred in 77% of patients with LN and 42% without LN (p=0.0001), whereas the frequency of the A allele was significantly lower (52% versus 77%) and the frequency of the G allele was significantly higher (48% versus 23%) in these 2 groups.

In contrast to these result, Aguilar, et al. [19] found no association between this polymorphism and susceptibility to SLE or to lupus nephritis. Also Sanchez, et al. [18], found no statistically significant difference between patients with and patients without lupus nephritis regarding distribution of MCP-1 genotypes.

The incidence of the most severe form of lupus nephritis (grade IV) was highest in association with GG genotype (33.3%) Table (6), but yet this was not significant statistically.

Also, no statistically significant difference was found regarding the presence of proteinuria, cells or casts in urine of patients of different genotypes (Table 7). These findings were similar to that of Chrisiane & Stephan [20] who reported that although there was a tendency toward increased proteinuria in AA homozygotes, the difference did not reach statistical significance.

There was no statistically significant difference between patients of different genotypes concerning the presence of anemia, leucopenia and thrombocytopenia (Table 7). This is similar to the results of Wallach, et al. [21] who found that the three parameters did not vary significantly with different MCP-1 genotypes. On the other hand, Gilbert, et al. [22] found a significant correlation between MCP-1 genotype and the incidence of leucopenia in SLE patients.

In our study although most patients (95.2%) have positive ANA, there was no significant difference among patients of the three genotypes. Gilbert, et al. [22] was in agreement with these findings, as he reported no significance difference between MCP-1 genotypes in the incidence of positive ANA tests.

Nearly, two thirds of patients (75%) are positive for anti-dsDNA Abs, yet the difference between MCP-1 genotypes was not statistically significant, which is in concordance with Sanchez, et al. [18] who reported similar results. As regards serum complement levels, 50% of GG cases compared to 57.1% of AA cases had low serum levels, yet this was not statistically significant (Table 7).

On the contrary, Sanchez, et al. [18] reported a significant correlation between the presence of G allele and low serum complement levels. In accordance to our findings, Marco, et al. [1] did not find any significant correlation of MCP-1 genotypes with any of all other ACR SLE criteria or other organ involvement, such as the CNS, joint, lung, hematologic system or with the WHO histologic class of nephritis.

Increased concentrations of MCP-1 in the diseased kidney may be an important factor contributing to increased production of cytokines and adhesion molecules [20].

MCP-1 is consistently found at high levels in the urine of patients with active LN. Moreover urinary MCP-1 levels decline with treatment of nephritis and they are not elevated in non-lupus forms of glomerulonephritis [11,12].
Urinary MCP-1 levels in this study were significantly higher in SLE patients 4.6 (1.3-15.4) compared with controls 1.4 (1.0-2.1) ($p=0.0001$) (Fig. 5-A). SLE patients with LN had significantly higher MCP-1 levels 6.0 (1.8-21.3) than did SLE patients without LN 2.4 (1.1-4.4) ($p=0.02$) (Fig. 5-B & Table 8). This indicates that the difference in urinary MCP-1 levels detected between the entire SLE and controls was primarily due to patients who had LN. Our findings were in accordance with Marco, et al. [1] who found that MCP-1 levels were significantly higher ($p=0.0001$) in SLE patients (mean ± SD 968.2±388.2 units) compared with controls (46.8±3.5 units). SLE patients with LN had significantly higher ($p=0.0001$) MCP-1 levels (1,732±388.8 units) than did SLE patients without LN (161.3±35.4 units).

In this study there was a negative correlation between urinary MCP-1 levels and serum creatinine ($p=0.004$) and a positive correlation between urinary MCP-1 levels and proteinuria ($p=0.000$) which also coincide with the results of Marco, et al. [1], Christiane & Stephan [20] and Kim, et al. [10]. Rovin, et al. [13] concluded that urinary MCP-1 is a sensitive and specific biomarker of renal SLE [23].

In conclusion: Our data showed that the A/G and G/G MCP-1 polymorphisms were associated with a greater likelihood of renal disease in SLE patients. Urinary levels of MCP-1 may be a useful marker for the early detection of LN in SLE patients.

**Recommendations:**

Screening for MCP-1 polymorphisms may be applied to determine the relative risk of developing renal disease in a patient with an established diagnosis of SLE without overt renal disease. Patients in high-risk categories may then be monitored more closely, or a renal biopsy may be suggested.

Measuring urinary MCP-1 levels longitudinally during treatment of LN and correlating urinary MCP-1 levels directly with the biopsy findings will help to further define the potential role of this marker in the management of LN.

**References**


