Evaluating Galectin-3 (LGALS3) +191 gene variant and serum Galectin-3 levels in Egyptian children with familial mediterranean fever

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Abstract---Background: The most important and devastating complications of Familial Mediterranean fever (FMF) is renal amyloidosis, usually affecting the kidneys leading to end stage renal failure. FMF-related renal amyloidosis needed to be diagnosed early. Optimal colchicine dose is effective in prevention and reversing renal amyloidosis. Aim of the work: to evaluate serum galectin-3 level and its gene polymorphism (LGALS3 191 C>A) as a marker of proteinuria and subclinical inflammation in Egyptian children and adolescents with FMF. Methods: Fifty FMF patients in attack free period and 40 healthy children were included as a control group. Serum levels of galectin-3 were measured, Galectin-3 (LGALS3) c.191 C>A (rs4644) gene variant was investigated and morning spot urine was collected for determination of albumin / creatinine ratio (ACR). Results: Serum Galectin-3 levels were significantly higher in FMF patients than control group, P < 0.03. Regarding genotype and allele distribution of (LGALS3) c. 191 C>A
polymorphism there was statistical significant difference between cases
and control, 13 patients (42%) had CC and the remaining 37 patients
(63%) were CA. The control had 18 child (58%) with CC compared to 22
child with CA (37%) and BUN level was higher among CC type of
Galectin-3 (LGALS3) c.191 C>A. Conclusion: increasing levels of galectin-
3 in FMF children in attack free period. Galectin-3 might be a strong
marker reflecting chronic subclinical inflammation in patients with FMF.

**Keywords**---FMF, Galectin-3, Proteinuria, Renal affection, Single
Nucleotide Polymorphism.

**Introduction**

Familial Mediterranean fever (FMF) is an inherited disorder characterized by
recurrent, irregular, and self-limited attacks of chest and abdominal pain
associated with fever [1]. The gene responsible for FMF is mapped on the short
arm of 16p 13.3. It is also designated MEFV (ME for Mediterranean and FV for
fever). Approximately 70% of patients with clinical manifestations of FMF are
heterozygous and the most common missense mutation is M694V. Genetic testing
for the FMF gene confirms the diagnosis of FMF [2].

The most common and pernicious complication of FMF is renal amyloidosis,
usually affecting the kidneys, leading to end-stage renal failure. Proteinuria is the
most common presenting feature of this complication [3]. Galectin-3 is a b-
galactoside-binding lectin, highly expressed in activated macrophages, and has
regulatory functions in inflammation, fibrosis and tumorigenesis. It plays a role in
chronic kidney disease [4]. Elevated galectin-3 levels were also present in patients
with Chronic Heart Failure [5].

Human galectin-3 gene (LGALS3) is located on chromosome 14q21. It is
composed of six exons that encode a protein of 32 kDa. It was implicated that the
 genetic polymorphisms in galectin-3 gene may contribute to development of
rheumatoid arthritis [6]. Role of Galectin-3 in pathogenesis of FMF remains to be
determined. Further evaluation of the role of the Galectin-3 genetic variants and
serum Galectin-3 in proteinuria and inflammation in children with FMF needs to
be studied thoroughly [7].

**Aim of the Work**

The aim of the study is to evaluate serum galectin-3 level and its gene
polymorphism (LGALS3 191 C>A) as a marker of proteinuria and subclinical
inflammation in Egyptian children and adolescents with FMF in attack free period.

**Patients and Methods**

This study included 50 Egyptian children and adolescents diagnosed as having
FMF and following up in the Pediatric Rheumatology Clinic of Specialized
Pediatric Hospital, Cairo University from the period of November 2014 to October
2015. Patients were diagnosed according to the new FMF criteria [8].
Control group: included 40 healthy children and adolescents. They were matched for age and sex with patients, coming for regular follow-up at General out-patient clinics.

Ethical approval: The study was approved by the Ethical Committee of Faculty of Medicine, Cairo University (ethical clearance number, I-150314). A written consent was obtained from all studied patients.

Inclusion criteria: The study included patients having FMF according to the new FMF criteria (8), age of disease onset before 18 years, patients are in attack free period “at least 2 weeks from the end of FMF attack period according to the physical and clinical symptoms” (9).

Exclusion criteria: patients with acute FMF attack, associated autoimmune disorders, associated diseases including diabetes mellitus, heart failure, hypertension and cancer and patients on the following medications (oral anti-diabetic agents, anti-hypertensive drugs, anti-lipidemic agents and glucocorticoids).

Funding: No funding was obtained for this study.

Methodology in details:

Fifty FMF patients who were recruited from regular attendance of Pediatric rheumatology clinic were subjected to the following:

1. Demographic data collection (gender, age, consanguinity of parents and family history of FMF/ renal disease)
2. Full clinical history and examination including:
   - Age at onset of FMF
   - Age at diagnosis
   - History of the presenting features (typical attacks of fever, abdominal pain, chest pain, joint affection, skin affection, muscle pain, scrotal pain and history of vomiting to exclude peritonitis)
   - History of renal affection (proteinuria or hematuria)
   - History of appendectomy
   - Assessment of clinical pattern of the disease: -
     - Disease duration
     - Duration of FMF attacks
     - Colchicine duration and adherence
     - Response to colchicine assessed by FMF-50 score by Ozen et al. (10),
     - Disease severity score assessed by severity score by Mor et al. (11),
     - Presence of MEFV gene mutation and its type.

Laboratory investigations: -

Blood samples were drawn for: -
A-CBC evaluation (Hb -TLC- PLT) by using automated haematology analyzer “automated counter “
B -CRP with titre, ESR, BUN, creatinine.
C-Serum levels of galectin-3 were measured using enzyme-linked immunosorbent assay (ELISA) technique.

D-Galectin-3 (LGALS3) c.191C>A (rs4644) gene variant was investigated by PCR followed by RFLP technique.

E-Urine sample was collected for:
1-Urine analysis.
2-Albumin / creatinine ratio.

**Urinary Albumin / Creatinine ratio (ACR) technique:**

Albumin-to-Creatinine Ratio (ACR) is one of the two markers used to determine chronic kidney disease. ACR is defined as the ratio between albumin (reported in mg/dl) and creatinine (reported in g/dl). Single void morning urine sample was collected for evaluation of albumin / creatinine ratio in spot urine during attack free period.

Estimated ACR by using albumin and creatinine concentrations was established in the sample(s) using the formula:

\[
\frac{\text{Albumin (mg/dL)}}{\text{Creatinine (g/dL)}} = \text{ACR} \frac{\text{Albumin mg}}{\text{g Creatinine}}
\]

Normal: \(0 \leq \text{ACR} \leq 30 \text{ mg/g creatinine}\)
Micro-albuminuria: \(30 \leq \text{ACR} \leq 300 \text{ mg/g creatinine}\)
Proteinuria Clinical: \(\text{ACR} > 300 \text{ mg/g creatinine}\) (https://www.biovision.com)

**Serum galectin-3 using ELISA technique:**

Blood samples were drawn and serum separated in a centrifuge for 15 min at 1000 rpm and immediately transferred into 1 ml eppendorf tube and stored at \(-80^\circ\) C until analysis \((12)\).

**a-Test principle:**

The kit used double-antibody sandwich (ELISA) to assay the level of Human Galectin-3 in samples. galectin-3 was added to monoclonal antibody Enzyme well which is pre-coated with Human Galectin-3 monoclonal antibody, incubated, then, Galectin-3 antibodies labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then carried out incubation and washed again to remove the uncombined enzyme. Then chromogen solution A, B was added, the color of the liquid change into blue, And at the effect of acid, the colour finally became yellow. The chroma of colour and the concentration of Human substance Galectin-3 of sample were positively correlated.

**b-Summary procedure:**

1. **Reagents, samples and standards were prepared**
2. Prepared samples and standards, antibodies labeled with enzyme were prepared, reacting 60 mins at 37°C
Plate washed five times, chromogen solution A, B were added, reacting 10 mins at 37°C

Stop solution was added

The optical densit (OD) value was measured within 10 mins

c- Calculation

The standard concentration was taken as the horizontal, the OD value for the vertical, the standard curve was drawn on graph paper, the corresponding concentration was found out according to the sample OD value by plotting on the standard curve or using the straight line regression equation of the standard curve for sample galectin-3 concentration calculation.

d. Sensitivity and assay range

- **Sensitivity**: 0.186 ng/ml (the sensitivity of this assay was defined as the lowest protein concentration that could be differentiated from zero)
- **Assay range**: 0.2 ng/ml – 60 ng/ml
  
  (https://www.sunredbio.com)

Galectin-3 (LGALS3) c.191C>A (rs4644) gene variant was investigated by PCR followed by RFLP technique:
The Analysis was done in 5 main steps:
I) Extraction of genomic DNA from peripheral blood leucocytes of EDTA anticoagulated blood.
II) Amplification of the target amplicon.
III) Detection of PCR amplification products using agarose gel electrophoresis.
IV) The amplified products were digested with the NcoI restriction enzyme.
V) After digestion the digested products were analyzed by electrophoresis on agarose gel for determination of variants.

(www.intronbio.com© 2012 INTRON Biotechnology)

Detection of LGALS3 (rs4644) Variant:
LGALS3c. 191C>A genotypes were identified according to the number and fragment length of detected bands on agarose gel using ultra-violet transillumination as shown in Table (1).

Table (1): The identified bands after digestion with NcoI restriction Enzyme

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of bands detected</th>
<th>Size of bands fragment length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>Two bands</td>
<td>443 bp</td>
</tr>
<tr>
<td>(Reference Homozygous Genotype)</td>
<td></td>
<td>110 bp</td>
</tr>
<tr>
<td>CA</td>
<td>Four bands</td>
<td>443 bp</td>
</tr>
<tr>
<td>(Heterozygous Genotype)</td>
<td></td>
<td>254 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>189 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110 bp</td>
</tr>
</tbody>
</table>
Statistical analysis

The statistical analysis was done using SPSS v22.0 IBM statistical package for social sciences (IBM, 2013). IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The significance level was set at \( p<0.05 \) & marked with significant (S), while highly statistical significance was set at \( p<0.01 \) & marked with highly significant (HS). The statistical insignificance was set at \( p>0.05 \) & marked by non-significant (NS).

Results

Out of 90 children which were included in the present study, 50 patients were diagnosed as having FMF according to the new FMF criteria and who had been using colchicine. Control group had no known disease, they were coming for pre-operative clinical and laboratory assessment for elective surgeries.

There were no statistical difference between patients with FMF and healthy individuals in terms of age 7(5-10) vs 8 (6-11), respectively. And also we did find male / female ratio between patients with FMF and controls (M/F=27/23 vs M/F=29/11), respectively. There was no statistical significant difference between the serum Galectin-3 levels regarding clinical and laboratory data illustrated in Table (2).

Table (2): Comparison between Serum Galectin-3 level and demographic, clinical & laboratory variable

<table>
<thead>
<tr>
<th>Demographic and clinical data</th>
<th>S.galectin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at time of study</td>
<td>0.38</td>
</tr>
<tr>
<td>Age at 1st symptom</td>
<td>0.28</td>
</tr>
<tr>
<td>Age at Diagnosis</td>
<td>0.28</td>
</tr>
<tr>
<td>Colchicine duration</td>
<td>0.83</td>
</tr>
<tr>
<td>Disease duration</td>
<td>0.77</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>0.4</td>
</tr>
<tr>
<td>Total leucocytic count (×10³/ul)</td>
<td>0.35</td>
</tr>
<tr>
<td>Platelets (×10³/ul)</td>
<td>0.06</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.86</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>0.68</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>0.76</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.23</td>
</tr>
<tr>
<td>Urinary albumin/creatinine ratio (mg/g)</td>
<td>0.36</td>
</tr>
</tbody>
</table>
Blood urea Nitrogen (BUN) level showed statistical significant higher values among LGALS3 CC genotype when compared to CA genotype (21.07±5.09, 17.1±5.2 respectively, p value=0.01).

**Table (3):** Levels of various Laboratory data among LGALS3 c. 191C>A variants

<table>
<thead>
<tr>
<th>Variable</th>
<th>LGALS3 c. 191 C&gt;A variants</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC genotype</td>
<td>CA genotype</td>
</tr>
<tr>
<td>Disease duration</td>
<td>2 (1 - 4)</td>
<td>3 (1.5 - 4)</td>
</tr>
<tr>
<td>Total leucocytic count (×10^3/ul)</td>
<td>6.5 (4.7 - 9.2)</td>
<td>7.5 (5.7 - 10.8)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.4±0.9</td>
<td>12.2±1.2</td>
</tr>
<tr>
<td>Platelets (×10^3/ul)</td>
<td>280.4±71.9</td>
<td>291.2±75.5</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.7 (2.05 - 8.6)</td>
<td>4.6 (2.6 - 5.8)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>26 (14.5 - 36)</td>
<td>26 (16 - 38)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>21.07±5.09</td>
<td>17.1±5.2</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.5 (0.5 - 0.6)</td>
<td>0.6 (0.5 - 0.6)</td>
</tr>
<tr>
<td>Urinary Albumin/creatinine ratio (mg/gm)</td>
<td>18.2 (16.2 - 32.7)</td>
<td>26.3 (18.8 - 35.1)</td>
</tr>
</tbody>
</table>

Values are represented in median (25TH – 75TH percentile) and mean (±SD)

Serum Galectin-3 levels were significantly higher in FMF patients than the control group with median & IQR of 20.5 (11.8-129.5) and 12.2 (3.1-66) ng/ml respectively (P=0.03). There was no statistically significant difference between the serum Galectin-3 levels regarding clinical and laboratory data. Regarding genotype distribution and allele frequencies of Galectin (LGALS3) c. 191 C>A variant there was statistically significant difference between cases and control, 13 patients (42%) had CC and the remaining 37 patients were C/A (63%). The control had 18 cases (58%) with CC compared to only 22 cases with C/A (37%).

There was statistically significant association between LGALS3 c.191C>A variants and MEFV gene mutations, where most patients with homozygous (67%) MEFV gene mutations had a LGALS3 CC genotype while most of the patients with heterozygous (81%) and compound heterozygous (72%) MEFV mutations had a LGALS3 CA genotype (P=0.04).
Blood urea Nitrogen (BUN) level showed statistically significant higher values among LGALS3 CC genotype when compared to CA genotype (21.07±5.09, 17.1±5.2 respectively, p value=0.01). Serum galectin-3 levels showed statistically significant difference between LGALS3 CC & CA genotypes in FMF patients where serum galectin-3 levels was lower in CC genotype patients than in CA genotype patients showing a median of 132.5 (10.8 – 165.2) and 18.7 (12.3 – 52.2) ng/ml respectively (P=0.02).

To estimate the reference value to diagnose case or control according to serum galectin-3 we perform the ROC curve analysis as shown in figure (2). The area under the curve was 0.66. At high sensitivity (ability to detect all positive including false positive) 98% and low specificity the cutoff value for S.galactin-3 by ELISA is 4.4. While at high specificity (ability to detect negative cases) is 81.3 (sensitivity 30% - specificity 80%).
Figure (2): Roc curve for the S. Galectin-3 according to case / control estimate.

**Discussion**

The main result of the study is that serum galectin-3 was significantly higher in FMF patients than in control group Yilmaz et al., (7). The most important and unwanted complication in FMF was development of renal amyloidosis. Appropriate dose of colchicine prevents renal amyloidosis in most of patients and is also effective in arresting and reversing renal amyloidosis. Galectin-3 is a reliable marker that can determine proteinuria and renal amyloidosis with the following mechanisms: (1) together with the amyloid which accumulated in glomerulus, advanced glycation end products (AGEs) and receptor for advanced glycation end products (RAGE) also accumulate and they correlate with amyloid deposits, galectin-3 is up-regulated and is operating as an AGEs receptor to afford protection toward AGE-dependent tissue injury and increase the expression of gal-3 acts as opposed to RAGE. So gal-3 level increases to prevent the damage caused by AGE; however it could be assumed that it could be not protective due to gal-3 resistance. (2) there is a positive correlation between glomerular amyloid accumulation and interstitial fibrosis in renal amyloidosis caused by FMF, galectin-3 is a pro-fibrotic marker, having a stimulatory effect on MQ migration, myo-fibroblasts accumulation/activation, and development of fibrosis in renal tissues. There is generally interstitial fibrosis on the basis on renal amyloidosis and gal-3 level might be increase depending on fibrosis. (3) persistence of chronic inflammation even if in attack free period may cause renal amyloidosis. In addition there is a positive correlation between degree of glomerular amyloid accumulation and inflammation. As a result, chronic inflammation in patients with proteinuria attack free FMF might be one of possible factors responsible for the increase of galectin-3 levels (4).
In our study there was no significant difference in terms of galectin-3 with age, duration of illness, adherence to colchicine, severity of the disease, CRP, ESR, BUN and creatinine in patients with FMF. CRP is an acute phase reactant that has no ability to determine subclinical inflammation and renal amyloidosis caused by FMF (6,7).

Galectin-3 (LGALS3 c.191C>A) variants showed significant difference in genotypic distribution between cases and control. The frequency of homozygous variant CC genotype was significantly higher in control (58%) as compared to cases (42%), Kaur et al., (6). This was suggestive evidence of an association in a Co-dominant model.

There was statistical significant difference between Galectin-3 (LGALS3 c.191 C>A) and MEFV gene mutation, where most of the heterozygous group were CA (81%) while most of the compound heterozygous were CA (72%) and most of the homozygous were CC genotype (67%), Sciacchitano et al., (13).

Also there were statistical difference between S.galectin-3 and its gene variants, where CA type showed higher median than those of CC type. Due to its regulatory role in immune response, inflammation and fibrosis (14).

Galectin-3 induces monocyte-macrophage differentiation, regulates apoptosis on T lymphocytes and inhibit B-lymphocyte differentiation into immunoglobulin secreting plasma cells. Galectin-3 play a “double-faced” role in regulation of autoimmune response. It may either dampen or foster the development of autoimmune disturbance, depending on which mechanisms are prominent in each disease (15).

The BUN level was higher among CC type of the Galectin-3(LGALS3 c. 191 C>A) compared to CA type mean 17.1±5.2 (P=0.01), this may be attributed to galectin-3 up regulation in acute kidney injury (5).

So, galectin-3 may have a potential to predict progressive decline in kidney function, Desmedt et al., (4). Galectin-3 is involved in the pathogenesis of several kidney diseases by promoting macrophage migration, myofibroblast activation and collagen synthesis.

**Limitations of this study**

First, this study enrolled a relatively small size sample size, second, we used spot morning urinary ACR for evaluation of proteinuria. Although the ACR correlates with 24-hr urine protein excretion, there are two major limitations of using random spot morning urine samples to quantity proteinuria: (a) the ACR is heavily influenced by the urine creatinine concentration (the denominator of the ratio) so ACR will underestimate or overestimate proteinuria, (b) urine protein excretion can vary though-out the day and from day to day. Third, AGE-RAGE and other inflammatory markers (Serum amyloid A (SAA), interleukin-1(IL-1)) were not measured except CRP and ESR. SAA is a better indicator of subclinical inflammation.
So the conclusion of this study warrants further confirmation with comparatively larger sample size, increasing levels of galectin-3 in patients with FMF might be indicator of subclinical inflammation, Galectin-3(LGALS3 c. 191 C>A) may be associated with susceptibility towards FMF.

Disclosure of interest:
The authors report there are no conflicts of interest.

Conclusion

The present study revealed increasing levels of galectin-3 in FMF children in attack free periods. This study emphasized that galectin-3 might be a strong marker reflecting chronic subclinical inflammation in patients with FMF. Multiple regression analysis between galectin-3(LGALS3191 C>A) variant among cases with other studied variables revealed that the strongest predictor was the level of BUN.

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References