Tumor Necrosis Factor Alpha 308 Gene Polymorphism and Serum Osteoprotegerin Inarteriovenous Fistula Dysfunction Inhemodialysis Patients

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Abstract: Introduction: The preservation of patent, well-functioning dialysis fistulas is one of the most difficult clinical problems in the long-term treatment of patients undergoing dialysis. Arteriovenous fistula (AVF) dysfunction remains a major contributor to the morbidity and mortality of hemodialysis patients. The goal is to identify a dysfunctional AVF early enough to intervene in a timely manner to either assist with the maturation process or to prevent thrombosis. Over the past years, numerous clinical studies have consistently reported higher serum levels of OPG in association with cardiovascular outcome including coronary artery disease (CAD), vascular calcification, advanced atherosclerosis. Genes encoding for TNF-alpha are potential genetic risk factors for atherosclerosis and have functional variants that regulate their expressions. Polymorphisms at the position -308 in the promoter region of the TNF-alpha gene have been implicated as risk factors for atherosclerosis. Factor V Leiden gene mutation (G1691A) has been recognized to be the most prevalent genetic risk factor for venous thrombosis. Aim of the work: The aim of the present work is to investigate TNF-alpha 308 gene polymorphism, factor V Leiden mutation (G1691A), and serum osteoprotegerin level in patients on maintenance hemodialysis suffering from vascular access dysfunction. Subjects & methods: The study included 60 end stage renal disease patients from Alexandria University Hospitals on maintenance hemodialysis who were divided into 2 groups; 40 patients with AVF dysfunction, 20 patients with normal functioning AVF, 20 age and sex matched healthy subjects of matched age & sex were used as a control group. Investigations include serum level of Osteoprotegrin by ELISA technique, Serum TNF-alpha-308G>A-polymorphism by real time PCR and factor V Leiden gene mutation was assessed using 5’ Nuclease assay as well as Doppler ultrasound for vascular access. Results: As regard TNF alpha 308 genepolymorphism there was statistically significant difference in the group with dysfunctional AVF compared to the other two groups while factor V laden gene mutation showed statistical significant difference in group with AVF dysfunction & control group while no significant difference between them and those with functioning AVF, OPG was higher in group with AVF dysfunction compared to those with functioning AVF & control group while there was no significant difference between functioning AVF & control group. Conclusion: both OPG and the genotype distribution of TNF-α -308 G > A can be used as potential markers on HD patients to detect AVF dysfunction while, the role Factor V Leiden gene mutations may need further research and investigations.

Keywords: AVF dysfunction, TNF-alpha- 308G>A-polymorphism; Osteoprotegrin; factor V laden gene mutation

1. Introduction

A successful functioning vascular access is the “lifeline” for a hemodialysis patient. Arteriovenous fistulae (AVF) are the preferred vascular access for hemodialysis patients, because once mature and functional, they require fewer interventions to maintain patency and develop fewer infections compared toarteriovenous grafts (AVGs). However, AVFs have higher rates of non-maturation and longer maturation times compared to AVGs.

The failure of a newly created AVF to mature and development of stenosis in an established AVF are two common clinical problems.

In AVF and AVG, the most common cause of this vascular access dysfunction is venous stenosis as a result of neointimal hyperplasia within the peri-anastomotic region of AVF or at the graft-vein anastomosis. There have been few effective treatments to-date for venous neointimal hyperplasia in part because of the poor understanding of the pathogenesis of venous neointimal hyperplasia. The goal of most researches is to identify a dysfunctional AVF early enough to intervene in a timely manner to either assist with the maturation process or to prevent thrombosis. The currently available tools include clinical evaluation, physical examination of the AVF, and surveillance tests but they are not enough so; there is a need to identify those patients at risk of development of AVF dysfunction.

Vascular inflammation plays a key role in the development of atherosclerosis in hemodialysis patients (HD). This role of vascular inflammation may be involved in the thrombosis process of AVF in this population. Tumor necrosis factor alpha (TNF-α) is a cytokine found in human atheromatous lesions and modulates inflammatory cellular response in atherosclerotic process. Genes encoding for TNF-α are potential genetic risk factors for atherosclerosis and have functional variants that regulate their expressions. Polymorphisms at the position -308 in the promoter region the TNF-α gene are associated with higher rate of transcription of gene and have been implicated as risk factors for atherosclerosis.
Osteoprotogerin (OPG), a member of the tumor necrosis factor receptor superfamily, is a soluble decoy receptor for the osteoclast differentiation factor receptor-activator of nuclear factor kB ligand (RANKL) that inhibits interaction between RANKL and its membrane-bound receptor RANK. The RANKL/OPG/RANK axis has been shown to regulate bone remodeling[11] and was more recently found to be linked to the development of atherosclerosis and plaque destabilization.[12] In observational studies, elevated circulating OPG levels have been associated with prevalence and severity of coronary artery disease, cerebrovascular disease, and peripheral vascular disease. Circulating OPG levels are increased in patients with acute coronary syndrome, and enhanced expression has been found within symptomatic carotid plaques.[13-15]

Several inherited factors that predispose to thrombosis have been identified.[16] Factor V Leiden mutation (G1691A) has been recognized to be the most prevalent genetic risk factor for venous thrombosis. Factor V plays a major role in homeostasis by converting prothrombin to thrombin. [17]

So, this study was conducted with the aim to investigate TNF-α308 gene polymorphism, factor V Leiden mutation (G1691A), and serum osteoprotogerin level in patients on maintenance hemodialysis suffering from vascular access dysfunction.

2. Patients & Methods

The study included 60 end stage renal disease patients on maintenance hemodialysis who were recruited from dialysis units of Alexandria University hospitals. The patients were divided into two groups 40 patients with AVF dysfunction & 20 patients with normal functioning AVF. 20 age and sex matched healthy subjects were included as a control group.

Patients with history of diabetes mellitus, coronary artery diseases, bone disease and autoimmune diseases were excluded from the study. Also, hypertensive, obese, and those taking anti-inflammatory drugs were excluded.

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and an informed consent was obtained from each patient and control in the study.

Each subject had medical history taken as well as, full clinical examination stressing on vascular access (The flow, presence of thrill, aneurism, singes of inflammation and obstruction).

Fasting blood samples were collected for serum cholesterol, serum triglyceride, low density lipoprotein (LDL), high density lipoprotein (HDL), complete blood count, renal function tests (blood urea, serum creatinine), Serum calcium level, Serum phosphate level, Intact parathyroid hormone level (iPTH), high sensitive CRP (hs-CRP) using standard laboratory protocols.

Osteoprotogerin[18]

Detection of serum level of Osteoprotogerin was done using sandwich enzyme linked immunosorbent assay (ELISA) technique (DRG International, Inc. USA)

TNF-alpha- 308G>A polymorphism and Factor V Leiden mutation polymorphism[19, 20]

Genomic DNA was extracted from whole blood by standard methods using the Pure Link® DNA kit (life technologies,CA, USA). Checking DNA quality and quantity was performed using Nano Drop (Thermo Scientific, USA). All samples were stored at - 20°C till further analysis.

Samples were genotyped for TNF-alpha- 308G>A-polymorphism and Factor V Leiden mutation using a TaqMan 5”-allele discrimination Assay-By-Design method (Applied Biosystems, Foster City, CA). The genotyping was performed on Rotor-Gene Q (Qiagen, Hilden, Germany).

TaqMan allelic discrimination was performed in 25 μL reaction volume containing the Universal 2x TaqMan® Master Mix (Applied Biosystems, Foster City, CA, USA), and the 20X Assay ready-made stock for both genes. Thermal cycling profile included an initial step at 95 °C for 10 min followed by 40 cycles of 95°C for 15 sec (denaturation step) and 60 °C for 1 min (annealing(extension).

Radiological study

Doppler ultrasound was done for assessment of AVF function and presence of thrombosis, calcification, obstruction or presences of steal phenomena using Siemens prima ultrasound system.

Statistical analysis of the data: [21]

All calculations were performed on a personal computer with IBM SPSS software package (version 20.0) for Windows.[22] Qualitative data were presented as numbers (n) and percentages (%) was compared using Chi square or Fisher Exact test. Quantitative data were presented as means and standard deviation (SD). Comparison between the means of quantitative variables was performed using the one-way ANOVA (F-test). While abnormally quantitative data expressed in Median (Min. – Max) and was compared using Mann Whitney or Kruskal Wallis test.

The correlations between different variables were evaluated by Pearson or Spearman correlation coefficients according to the distribution of variables (continuous or discontinuous quantitative variables respectively). P value ≤ 0.05 was accepted as statistically significant.

3. Results

Clinical and biochemical characteristics of the study subjects are presented in table (1)

Serum calcium, serum phosphorus, serum intact parathormone level, Total cholesterol, triglyceride, LDL, HDL, have a significant difference all through the three groups (p<0.001).

OPG level was higher in the group with AVF dysfunction compared to both the group with functioning fistula and controlgroup (p= and respectively), while there was no significant difference between functioning AVF & control group. (p<0.001). table(2).
Also we found hsCRP positively correlated to serum OPG level (p=0.004).

Regarding TNF alpha 308 gene polymorphism, in patients with AVF dysfunction 27 patients were heterozygous which was significantly higher than group with functioning AVF where only 5 patients were heterozygous while in control group there was 8 subjects heterozygous, and no significant difference between patients with functioning AVF & control group.(p=0.005).

Factor V laden gene mutation 16 patients were wild in functioning AVF & 29 patients with dysfunction and 13 in control group while heterozygous there was 1 in functioning AVF ,9 patients with dysfunction & zero in control group .as regard gene mutation there was 3 patients mutant in functioning AVF ,2 patients in dysfunctioning fistula and 71n control group which means that there was no significant difference between dysfunction AVF & functioning one.(p=0.104) while there was significant difference between group with dysfunction & control group(p=0.002).

Table (2)

Doppler ultrasound for AVF showed 9 cases with calcification and 11 patients thrombosis & calcification with average flow volume.(mean±SD 968.60 ± 115.07 ml/min).

4. Discussion

Inflammation proposed to play an important role in the initiation and progression of atherosclerosis in ESRD, but may also play a significant role in vascular access stenosis and thrombosis.[23,24]

In the present study it was found that hsCRP was significantly higher in the group with AVF dysfunction compared to those with normal functioning AVF this supports the role of inflammation in fistula dysfunction.

Zadeh et al.[25] studied the role of inflammation in failure of AVF and evaluate the hematologic and inflammatory biomarkers in early AVF failure. They included 110 ESRD patients, whom were undertaken AVF creation, divided in two groups and they found CRP was positive in 34 patients (61.8%) with unsuccessful fistula function, while only 4 (7.3%) of those with successful AVF had positive CRP and the rest had negative CRP.

Tumor necrosis factor-α (TNF-α) is a cytokine that initiates the inflammatory cascade and may contribute to inflammation in renal failure. Promoter polymorphisms of cytokine genes regulate the expression of cytokine and can affect the transcriptional activity.[26] A single base pair polymorphism which is located at -308 G/A in TNF-α gene is associated with a state of high TNF-α production and susceptibility to several diseases. It is responsible for inter individual differences in TNF-α production.[27]

In this study, patients with AVF dysfunction are higher heterozygous regarding the gene polymorphism compared to patients with normal functioning AVF and control subjects and no significant difference between patients with functioning AVF & control group, this mean that genetic polymorphism may paly role in AVF dysfunction.

Sener EF et al.[28] reported that genotype distribution of TNF-α -308 G > A in patients with AVF thrombosis was significantly different from the patients without thrombosis (p = 0.008) and propose that TNF-α -308 G > A genotype may be a potential genetic marker on HD patients with AVF thrombosis.

Buraczynska M et al.[29] also studied the A allele of the TNF-308 polymorphism and found it more frequent in the ESRD group than in control individuals. The odds ratio (OR) for the risk allele was 2.05. In the subgroup of ESRD patients with CVD, the OR was 5.76, relative to ESRD patients without CVD. They reported that the A allele of the TNF-308 polymorphism is associated with CVD in hemodialysis ESRD patients. And if confirmed in prospective studies, it may be a predictor of increased susceptibility to CVD in these patients.

In agreement with the current work, Girndt et al. [30] reported that inflammation is associated with enhanced development of atherosclerosis, endothelial dysfunction, and vessel wall proliferation. Therefore, genotypes of pro-inflammatory cytokines might influence the patency of AVF or AVGs by modifying vessel wall remodeling. A large array of genetic polymorphisms within pro-inflammatory cytokine genes is known, many of them with relevant functional consequences. To date, only the tumor necrosis factor-a (TNF-a) gene has been studied in relation to fistula patency.[31]

Regarding factor V Leiden gene mutation there was no difference between the group with AVF dysfunction and those with normal AVF as (p=0.104) while there was significant difference between group with AVF dysfunction & control group as (p=0.002).

Verschuren JJ et al. [32] studied 479 incident hemodialysis patients between January 1997 and April 2004. Follow-up lasted 2 years or until AVF failure, in total, 207 (43.2%) patients developed AVF failure. After adjustment, two SNPs (single nucleotide polymorphism) were significantly associated with an increased risk of AVF failure. Patients with Factor V Leiden had a hazard ratio of 2.54 (1.41 to 4.56) to develop AVF failure. The other SNPs were not associated with AVF failure.

Emirogullari E.F. et al. [33] had studied 31 HD patients with AVF thrombosis and 51 HD patients without AVF thrombosis and reported that there were no significant differences between HD patients with and without AVF thrombosis in terms of Factor V Leiden and Prothrombin G20210A mutations this result supports ours.
also in our study we did not find any significant difference between the group with AVF dysfunction and those with normal functioning AVF as regard factor V Leiden gene mutation.

Rios et al. stated in a cross-sectional study included 195 patients with end stage renal disease (ESRD) on HD for more than six months, that HD patients were allocated into two groups according to the occurrence (cases, N = 46) or not (controls, N = 149) of previous vascular access thrombosis. FV Leiden and prothrombin gene mutations were studied. They found that vascular access thrombosis mutation and ABO blood group were not associated with vascular access thrombosis, whereas G20210A mutation in the prothrombin gene was significantly higher in patients with vascular access thrombosis and independently associated with this complication (OR = 12.0; CI 95% = 1.8–83.5; p = 0.012).

In the current study, OPG was significantly higher in group with AVF dysfunction than those with functioning AVF and control group while there was no significant difference between functioning AVF and control group (p = 0.001).

Kim HR et al. studied serum fetuin-A, OPG and Hsp70 concentrations in 64 HD patients and studied the association of fetuin-A, OPG and Hsp70 with the degree of AVF stenosis using ultrasonography. They found that the degree of AVF stenosis was positively correlated with OPG levels (r = 0.411, p = 0.001) and OPG/fetuin-A ratio (r = 0.375, p = 0.002). The levels of OPG and OPG/fetuin-A ratio were statistically higher in the group of AVF stenosis ≥11.5%.

Morena M et al. studied 128 HD patients with internal AVF and followed up for two years by Doppler ultrasonography and serum albumin, prealbumine, C-reactive protein, orosomucoid, calcium, phosphorus, parathyroid hormone, bone-type alkaline phosphatase, OPG and receptor activator of nuclear factor κ B ligand were measured. The results confirm that vascular access (VA) thrombosis occurs more frequently upon preexisting stenosis, also demonstrate that mineral metabolism disorders, compared to inflammation, may contribute to VA dysfunction leading to thrombosis which agree with our work as we found that serum Ca level was lower while serum phosphorus level, OPG and iPTH were statistically higher in the group with AVF dysfunction compared to those with normal functioning AVF & control group. This means that mineral metabolism may play a role in the vascular access failure or dysfunction.

5. Conclusions

Serum OPG level and serum TNF-alpha-308G>A-polymorphism play a role in the arteriovenous fistula dysfunction in HD patientseven may be early predictors of the fistula dysfunction, while factor V Leiden gene mutation needs further investigations.

6. Recommendations

We need further investigation by measuring serum level of TNF & its receptor and its role in AVF dysfunction plus genetic polymorphism also need further research for the role of coagulation system in fistula dysfunction in patients on HD.

References


### Table 1: Biochemical results of studied groups

<table>
<thead>
<tr>
<th>Dysfunction</th>
<th>Control (n=20)</th>
<th>Yes (n=40)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.0 ± 11.91</td>
<td>55.55 ± 10.57</td>
<td>49.45 ± 4.90</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>11.52 ± 3.03</td>
<td>10.93 ± 2.45</td>
<td>-</td>
</tr>
<tr>
<td>BUN</td>
<td>26.50 ± 9.53</td>
<td>26.13 ± 9.85</td>
<td>-</td>
</tr>
<tr>
<td>HB</td>
<td>9.82 ±0.71</td>
<td>9.49 ±0.76</td>
<td>13.08 ± 0.85</td>
</tr>
<tr>
<td>Ca</td>
<td>9.12 ± 1.18</td>
<td>8.08 ± 1.08</td>
<td>10.20 ± 0.34</td>
</tr>
<tr>
<td>Ph</td>
<td>5.96 ± 1.75</td>
<td>7.57 ± 1.95</td>
<td>3.18 ± 0.48</td>
</tr>
<tr>
<td>iPTH</td>
<td>235.59 ± 224.54</td>
<td>416.53 ± 446.17</td>
<td>46.35 ± 11.13</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>172.15 ± 44.93</td>
<td>205.48 ± 43.90</td>
<td>130.80 ± 27.43</td>
</tr>
<tr>
<td>TG</td>
<td>128.15 ± 53.19</td>
<td>190.93 ± 115.1</td>
<td>92.92 ± 8.35</td>
</tr>
<tr>
<td>LDL</td>
<td>41.60 ± 10.0</td>
<td>8.60 ± 5.23</td>
<td>-</td>
</tr>
<tr>
<td>HDL</td>
<td>62.22 ± 10.0</td>
<td>8.60 ± 5.23</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 2: Comparison between groups regarding TNF-α gene, factor V mutation and OPG

<table>
<thead>
<tr>
<th>Dysfunction</th>
<th>Control (n=20)</th>
<th>Yes (n=40)</th>
<th>Test of Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF alpha Wild</td>
<td>15</td>
<td>75.0</td>
<td>12</td>
</tr>
<tr>
<td>Hetero</td>
<td>5</td>
<td>25.0</td>
<td>8</td>
</tr>
<tr>
<td>Sig. bet. grps.</td>
<td>p₁&lt;0.001, p₂=0.042</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FV Wild</td>
<td>16</td>
<td>80.0</td>
<td>29</td>
</tr>
<tr>
<td>Hetero</td>
<td>1</td>
<td>5.0</td>
<td>9</td>
</tr>
<tr>
<td>Mutant</td>
<td>3</td>
<td>15.0</td>
<td>2</td>
</tr>
<tr>
<td>Sig. bet. grps.</td>
<td>p₁=0.104, p₂=0.269, p₃=0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG Min. – Max.</td>
<td>21.70 – 190.0</td>
<td>103.40 – 468.40</td>
<td>58.0 – 220.0</td>
</tr>
<tr>
<td>Mean ± SD.</td>
<td>89.42 ± 41.76</td>
<td>231.76 ± 91.32</td>
<td>86.0 ± 35.09</td>
</tr>
<tr>
<td>Median</td>
<td>83.40</td>
<td>197.0</td>
<td>82.0</td>
</tr>
<tr>
<td>Sig. bet. grps.</td>
<td>p₁&lt;0.001, p₂=0.878, p₃&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

χ²: Chi square test
MC: Monte Carlo for Chi square test
F: F test (ANOVA), Sig. bet. grps was done using Post Hoc Test (LSD)
p₁: p value for comparing between No and yes
p₂: p value for comparing between No and control
p₃: p value for comparing between yes and control
*: Statistically significant at p ≤ 0.05
Table 3: Relation between dysfunction with TNF alpha gene, factor V mutation and OPG

<table>
<thead>
<tr>
<th>Dysfunction</th>
<th>No(n=20)</th>
<th>Yes(n=40)</th>
<th>Test of Sig.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><strong>TNF alpha</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>15</td>
<td>75.0</td>
<td>13</td>
<td>32.5</td>
</tr>
<tr>
<td>Hetero</td>
<td>5</td>
<td>25.0</td>
<td>27</td>
<td>67.5</td>
</tr>
<tr>
<td><strong>Fv</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>16</td>
<td>80.0</td>
<td>29</td>
<td>72.5</td>
</tr>
<tr>
<td>Hetero</td>
<td>1</td>
<td>5.0</td>
<td>9</td>
<td>22.5</td>
</tr>
<tr>
<td>Mutant</td>
<td>3</td>
<td>15.0</td>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>OPG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>21.70 – 190.0</td>
<td>103.40 - 468.40</td>
<td>χ² = 4.051</td>
<td>MC, p= 0.105</td>
</tr>
<tr>
<td>Mean ± SD.</td>
<td>89.42 ± 41.76</td>
<td>231.76 ± 91.32</td>
<td>8.277*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Median</td>
<td>83.40</td>
<td></td>
<td>197.10</td>
<td></td>
</tr>
</tbody>
</table>

χ², p: χ² and p values for Chi square test for comparing between the two groups
*: Statistically significant at p ≤ 0.05, MC: Monte Carlo for Chi square test, t: Student t-test