Risk Association between TNF-α-308 G>A and IL-6-174 G/C Polymorphisms and Recurrent Transient Ischemic Attacks

Hassan Salama and Enas Hammad

Departments of Neurology and Medical Microbiology & Immunology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Transient ischemic attack (TIA) is a brief cerebral ischemic incident. This study assesses the role of TNF-α and IL-6 single nucleotide polymorphisms (SNPs) as predictors of recurrent TIAs that have high risk of developing stroke. The current study enrolled 54 high risk (according to frequency of TIA) TIA (group1), 52 low risk TIA patients (group2) and 34 controls (group3). Polymerase chain reaction (PCR) for DNA amplification was done followed by restriction endonuclease analysis to detect SNPs of TNF-α-308 G>A and IL-6-174 G/C. TNF-α serum level was analysed by ELISA. Significant increase of TNF-α-308 G>A allele (group1 compared to group2 and control $P=0.0001$) and genotype TNF-α-308 AA ($P\leq0.05$) were detected. IL6 allele polymorphism revealed insignificant SNPs. The serum TNF-α was higher in group1 compared to control and group2 and as well in TNF-α-308 AA variant in high risk group ($P\leq0.05$). It is concluded that TNF-α-308 G>A SNP might have a role in predicting recurrent TIA with impact on preventive measures of stroke development.

Transient ischemic attack (TIA) is the occurrence of rapid loss of focal brain or monocular symptoms that continue for less than 24-hours. It is considered to be caused by insufficient cerebral or ocular blood flow as a result of arterial thrombosis, low flow or embolism which associated with arterial, haematological or cardiac diseases (Hatano, 1976).

Owing to advanced radiological scope in diagnosis of early stroke and time window of thrombolytic therapies, the propensity is strong to change time window of TIA to one hour. After one hour the patient is considered to be a high risk TIA with different management to prevent subsequent actual cerebral stroke risk (Albers et al., 2002).

Cytokines have dual rule in immune response to inflammation which is key element of the pathobiology of ischemic brain injuries (Lalouschek et al., 2006).

Either, as pro-inflammatory cytokine provokes or anti-inflammatory modulates immune response. Tumour necrosis factor-α (TNF-α) is one of the most potent pro-inflammatory cytokine that has a considerable role in the occurrence of ischemic brain injuries. It also carries functional polymorphisms (TNF-α-238 G/A and TNF-α-308 G/A) in its promoter region, which affect their transcription rate and plasma cytokine level (Castillo et al., 2003).

In addition the proinflammatory cytokine interleukin IL-6-174 G/C polymorphism has been implicated as unclear risk marker for ischemic brain etiopathogenesis (Tso et al., 2007). The prevention strategies both for risk prediction and potential intervention avert future brain ischemic events. Although genetic studies mentioned several genetic variants concerns as risk factors for ischemic or haemorrhagic stroke, the genetic determinants of these conditions remain deeply unknown for TIAs (Flossmann et al., 2004).

It is tentative if there is direct physiologic role between proinflammatory cytokine gene polymorphisms in promoter region and
regulation of their serum concentration level (Cui et al., 2012).

This study is conducted to evaluate the relation between TNF-α-308 G>A and IL-6-174 G/C polymorphisms and the risk of TIA recurrence in a sample of the Egyptian population and if the detected high risk polymorphisms have an impact on the serum expression level of their cytokine.

Patients and Methods

One hundred forty subjects were enrolled in this prospective cross-sectional study and divided into three groups, first group 54 patients with multiple TIA’s history with at least one recent attack (high risk TIA), second group 52 patients with one TIA over the last year (low risk TIA) and third control group includes 34 volunteers with no previous history of TIA.

All patients were subjected to thorough neurological history and examination, brain CT scan, ECG as well echocardiography to rule out valve disease with or without vegetation. Patients with history of recent definite ischemic stroke either clinical, radiological or cardiac valve diseases were excluded.

Genomic DNA extraction: Blood samples were collected using EDTA containing blood tubes. Genomic DNA was extracted from whole blood using the QIAmpDNA Mini kits TM (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer’s instructions. Briefly, 200 μL blood were added to 200 μL of sterile phosphate buffered saline then vortexed, followed by digestion with proteinase K and applied to 10 μL of sterile phosphate buffered saline then vortexed, followed by digestion with proteinase K and applied to silica gel spin columns. Columns were washed with the manufacturer’s supplied buffers and the total DNA was eluted in 200 μL elution buffer.

PCR-RFLP

1-TNF-α-308 G>A polymorphism

PCR-RFLP was used to detect TNF-α-308 G>A polymorphism, the following PCR primers were used: Forward primer 5’-TCCCCAAAAGAAATGGAGG CAATA- 3’ and Reverse primer 5’-GGTTTTTGAGGG CCATGAGACGTCTGCTGGGTG- 3’. PCR was conducted according to Petrişor et al., 2013 for 35 cycle of amplification (30 seconds each at 95°C, 30 seconds at 60°C and 1 minute at 72°C) (Petrişor et al., 2013). The amplification was preceded by an initial 12 minutes denaturation at 95°C and followed by 5 minutes final elongation containing at 72°C. PCR was carried out in a thermal cycler (Perkin Elmer, CT, USA).

One hundred ng of genomic DNA was amplified using 0.2 μM concentrations of each primer in a total volume of 50 μl PCR mix (EzWay PCR master mix, Koma Biotech, Korea) containing Taq DNA polymerase 1.25 units, 200 μM of each dNTP, and PCR reaction buffer final concentration of 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl. Five units of Neo I enzyme were added to the PCR amplification products for digestion and incubated at 37 °C for 40 min (Thermo Fischer Scientific Inc., MA, USA) and compared with 50 bp DNA molecular size standard DNA ladder. The resulted product were then separated on 2% agarose gel and visualized with UV light. Three possible banding patterns can be detected by electrophoretic analysis, each related to certain genotype: GG type homozygous (326 and 36 bp fragments), GA heterozygous genotype (362, 326 and 36 bp fragments) and AA genotype (362pb), (Figure 1).

2- IL-6-174 G/C polymorphism

The IL-6 promoter polymorphisms at position -174 were genotyped by performing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay as described previously (Abdel-Hady et al., 2009). Briefly, the following primer set were used for amplification of Interleukin-6 (-174) Forward 5’-TGACTTCAGCTTTACTCTTGT-3’ Reverse 5’-CTGATTGGAAACCTTATTAAG-3’

The cycling conditions used were, five cycles (96°C for 9 min, 1 min at 55°C and 72°C for 3 min) followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min with a final extension for 10 min at 72°C. Then, amplification product was digested with 5 units of Hin Iii (NiaIII) restriction endonuclease enzyme at 37°C for 40 min (Thermo Fischer Scientific Inc., MA, USA) and separated on 2% agarose gel using 50 bp DNA molecular size standard DNA ladder as a marker.

This digestion reveals a three allele polymorphism, allele GG homozygous genotype: 168 bp, GC heterozygous genotype 168 + 119 + 49 bp and CC homozygous genotype: two fragment of 119 bp + 49 bp (Figure 2).

TNF alpha ELISA

From all participants, venous blood samples (3 mL) were collected. Serum samples were stored at -20°C until analysed. Serum TNF-α immunoassay was determined according to the manufacturer’s instructions (ELISA: Ray Bio® Human TNF ELISA kit protocol, Ray Biotec, USA).
Statistical Analysis

Chi square test with Yates continuity correction were used for allele and genotype frequencies of studied polymorphisms; Fisher’s exact tests were applied if the expected frequency was less than 5. Odds ratios with 95% confidence intervals were determined. A $P$ value of $<0.05$ was considered to be significant.

Hardy-Weinberg equilibrium analysis was performed for each SNP by testing the observed genotype distribution against the expected distribution calculated on the base of the SNP allelic frequencies (to assess whether the tested SNPs change genotype distribution and allelic frequencies over generations or not). $P > 0.05$ (using a Chi-square test) was considered to signify equilibrium. Data were statistically analysed using the Statistical Package for Social Science program (SPSS version 15.0 for windows, Chicago, IL).

Figure 1. An ethidium bromide gel electrophoresis for TNF alpha -308 G/A gene polymorphism. Lane 1-50 bp reference marker, Lane 2 homozygous genotype AA; lane 3 heterozygous type GA, Lane 4 homozygous genotype GG.

Figure 2. An ethidium bromide gel electrophoresis for IL-6 -174 G/C gene polymorphism. Lane 1-50 bp reference marker, Lane 2 heterozygous type GC; lane 3 homozygous genotype CC, Lane 4 homozygous genotype GG.
Results

The enrolled characteristics of patient with TIA who fulfilled the inclusion criteria and controls are represented in (Table 1). Two allelic genotypes were tested; TNF-α-308 G>A and IL-6-174 G/C allele polymorphisms. The individual genotypes distributions of both polymorphisms were within Hardy-Weinberg equilibrium in the three studied groups.

The distribution of TNF-α-308 G>A and IL-6-174 G/C genotypes and allele frequencies in high risk, low risk TIA and control subjects is represented in Table 2 respectively.

Table 1. Characteristics of patients with TIA and controls.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=54)</th>
<th>Group 2 (n=52)</th>
<th>Group 3 (n=34)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ±SD)</td>
<td>60.4±11.6</td>
<td>61±11.9</td>
<td>59.7±12.8</td>
<td>NS</td>
</tr>
<tr>
<td>Gender % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>48.2 (26)</td>
<td>46.2 (24)</td>
<td>47.1 (16)</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>51.8 (28)</td>
<td>53.8 (28)</td>
<td>52.9 (18)</td>
<td></td>
</tr>
<tr>
<td>TIA site % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior circulation</td>
<td>31.5 (17)</td>
<td>13.5 (7)</td>
<td>-</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>Anterior circulation</td>
<td>68.5 (37)</td>
<td>86.5 (45)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Risk factors % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>74.1 (40)</td>
<td>57.7 (30)</td>
<td>47.1 (16)</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>DM</td>
<td>40.7 (22)</td>
<td>38.5 (20)</td>
<td>38.2 (13)</td>
<td>NS</td>
</tr>
<tr>
<td>AF</td>
<td>14.8 (8)</td>
<td>15.4 (8)</td>
<td>14.7 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>IHD</td>
<td>31.5 (17)</td>
<td>15.4 (8)</td>
<td>11.8 (4)</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>35.2 (19)</td>
<td>30.8 (16)</td>
<td>32.3 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Current smoking</td>
<td>37 (20)</td>
<td>34.6 (18)</td>
<td>35.3 (12)</td>
<td>NS</td>
</tr>
<tr>
<td>Carotid stenosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No stenosis</td>
<td>77.8 (42)</td>
<td>84.6 (44)</td>
<td>88.3 (30)</td>
<td>NS</td>
</tr>
<tr>
<td>Less than 50%</td>
<td>14.8 (8)</td>
<td>9.6 (5)</td>
<td>8.8 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>More than 50%</td>
<td>7.4 (4)</td>
<td>5.8 (3)</td>
<td>2.9 (1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Group 1: high risk TIA patients. Group 2: low risk TIA patients. Group 3: control. P> 0.5 is not significant (NS).

Table 2. Genotypes and allele frequency of TNF-α-308 G/A and IL-6-174 G/C polymorphism:

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>GG n (%)</th>
<th>GA n (%)</th>
<th>AA n (%)</th>
<th>G allele frequency</th>
<th>A allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>54</td>
<td>4 (7.4)</td>
<td>18 (33.3)</td>
<td>32 (59.3)</td>
<td>26 (24.1%)</td>
<td>82 (75.9%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>52</td>
<td>16 (30.8)</td>
<td>21 (40.4)</td>
<td>15 (28.8)</td>
<td>53 (51%)</td>
<td>51 (49%)</td>
</tr>
<tr>
<td>Group 3</td>
<td>34</td>
<td>10 (29.4)</td>
<td>16 (47.1)</td>
<td>8 (23.5)</td>
<td>36 (53%)</td>
<td>32 (47%)</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>54</td>
<td>17 (70.8)</td>
<td>21 (38.9)</td>
<td>16 (29.3)</td>
<td>55 (51%)</td>
<td>53 (49%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>52</td>
<td>14 (26.9)</td>
<td>22 (42.3)</td>
<td>16 (30.8)</td>
<td>54 (49%)</td>
<td>50 (51%)</td>
</tr>
<tr>
<td>Group 3</td>
<td>34</td>
<td>12 (35.3)</td>
<td>16 (47.1)</td>
<td>6 (17.7)</td>
<td>40 (59%)</td>
<td>28 (41%)</td>
</tr>
</tbody>
</table>
Frequencies of the GG, GA, AA genotypes of TNF-α-308 SNP were 29.4%, 47.1% and 23.5% in controls, 30.8%, 40.4% and 28.8% in low risk TIA and 7.4, 33.3 and 59.3 in high risk TIA respectively, the frequency of the A allele in high risk TIA (75.9%) was higher than that in the low risk TIA and controls (49% and 47% respectively). Statistically significant differences were found between patients with high risk TIA (group 1) compared to any of the two other subjected groups (low risk and controls )with respect to the -308 G>A genotype (homozygote, AA genotype) or allele distribution (A allele) [Odds ratio (95% confidence intervals): 3.3(1.83-5.9), P = 0.0001 and 3.5 (1.85-6.79), P= 0.0001 respectively] and [Odds ratio (95% confidence intervals): 10 (2.5- 3.2 and), P ≤ 0.05 and 8.5 (2.4- 30), P ≤ 0.05] respectively.

Moreover, when we compared the AA and GA genotypes against GG genotype as a reference, the correlation between TNF alpha SNP genotypes and TIA risk was tested in subgroup of high risk TIA subjects, high statistical significant difference of genotype TNF-α-308 AA [Odds ratio (95% confidence intervals): 8 (2.65- 24.17 and), P = 0.0002] was detected. While genotype GA revealed lower statistical significance [Odds ratio (95% confidence intervals): 4.5 (1.42- 14.72 and), P = 0.01].

The IL-6-174 genotypes as well as their allele polymorphisms were not significantly different in patients with high risk TIA compared to low risk or control [Odds ratio (95% confidence intervals): 1.38 (0.75- 2.5), P > 0.05 and 0.9 (0.52- 1.53), P > 0.05] respectively.

It was found that mean serum TNF-α level was significantly higher in the high risk TIA patients 158.32± 1.29.40 compared to low risk TIA 8.80± 5.88pg/ml or controls 7.20±2.74 pg/ml (P≤0.05). No significant difference was found in plasma TNF-α between low risk TIA patients and controls.

The relationship between TNF-α promoter polymorphism and its serum concentration was analysed subsequently in high risk TIA subjects. The genotype AA 233.36± 115.74pg/ml is associated with increased serum TNF-α levels (P<0.05) compared to those with GA 57.97±36.19pg/m and GG 9.69±5.63pg/ml.

**Discussion**

Although not many studies of increased risk of TIA recurrence association with proinflammatory cytokines polymorphisms exist (Wang et al., 2013) two valuable findings in the present study correlating TNF-α-308 G>A allele polymorphism with the frequency of TIA recurrence and clinical variables among all groups. First, results indicated that TNF-α-308 G>A allele polymorphism gene variant were found to be statistically significant in group 1 compared with other groups, second subgroup analysis showed inside high risk recurrent TIA group that homozygote individuals had high risk than wild and heterozygote patients (Lalouschek et al., 2006; Castillo et al., 2003; Wang et al., 2013).

The proinflammatory TNF-α acts on endothelial leukocyte adhesion molecule expression and increases the penetration of inflammatory cells in cerebral ischemia and might increase risk of recurrent ischemic attacks (Watters et al., 2013).

Different studies found a relationship between TNF-α polymorphisms and ischemic attacks. Moreover, several studies instituted TNF-α gene polymorphism (guanine to adenine transition at -308) to be a risk for cerebral ischemia (Lalouschek et al., 2006; Wang et al., 2013).

Others mentioned that they play a protective role against ischemic changes
(Watters et al., 2013). While other studies found no relation between TNF-α gene polymorphism and cerebral ischemia (Fava et al., 2010).

The current outcomes as regard IL-6 polymorphisms are in agreement with prior reports of no significant difference among TIA cases in relation to IL-6-174 G/C allele polymorphisms or in relation to control group (Yin et al., 2013). While other studies observed that G allele or GG genotype associated with high risk ischemic episode, others mentioned this genotype contributes a defensive role against it (Shenhar-Tsarfaty et al., 2010). Conversely, some studies found C allele and CC genotype correlated with ischemic attacks. Moreover, a few researchers instituted gene dissimilarity related to individual subtype for instance hypertensive and smokers (Greisenegger et al., 2003). These ambiguous outcomes might be explained by the fact that IL-6 has dual roles as pro- and anti-inflammatory functions with a complex transcriptional regulation. Adding together, IL6 may have different effects attributed to vascular biology dissimilarities and pathophysiology (Tso et al., 2007; Fava et al., 2010).

The different outcome of the current study could be explained by many reasons: first, relatively small and homogenous groups as regard age and ethnicity, significant interethnic variation in the frequencies of polymorphisms have been mentioned. Second, regardless of polymorphisms being within the probable frequencies, the undersized number of homozygous TNF-α and IL6 may limit the capability to evaluate the association with TIA recurrence (Weiner et al., 2014).

Anyhow, it is very difficult to rule out the possibility that other markers in linkage disequilibrium (LD) are causative factors, so further studies using restrictive follow up and other related cytokines are required to establish whether it is indeed the TNF-α and IL-6 locus or other genes in LD that are accountable for these associations. Longitudinal studies are needed to confirm the direct relation between IL-6 and TNF-α and inflammatory process that associated or preceded permanent or transient ischemia.

In present study, 75.9% patients who experienced recurrent TIA had TNF-α A allele frequency and 49% IL-6 C allele frequency which is greater in our study than in previous reports. Generally speaking, TNF-α and IL-6 bear functional polymorphism in promoter regions that reflected on their transcription and plasma levels (Watters et al., 2013; Yin et al., 2013). TNF-α is an upstream modulator of many inflammatory cytokines that have roles in TIA etiopathogenesis via atherogenesis, disorder of lipid metabolism and impaired endothelial integrity (Sempere et al., 1998).

It was observed reasonable TNF-α-308 G>A predictive accuracy in patients with recurrent TIA in contrast to other studies (Castillo et al., 2003; Wang et al., 2013). However, for clinicians faced with management decisions involving individual patients, we suggest that patient with TNF-α-308 G>A polymorphisms may be treated on an urgent basis. This may be the case particularly in younger patients. Taken together, the present study observed a contributory role played by TNF-α-308 G>A among TIA patients with high risk of stroke requiring rapid evaluation and treatment.

Comparative analysis for TIA among females versus control women and TIA men versus control males, did not disclose any statistical significant. Hypertension and ischemic heart disease revealed significant statistical relation with high risk TIA and TNF-α-308 G>A allele polymorphism but not IL-6 (Eguchi et al., 2009; Garg et al., 2013).
On studying serum TNF alpha (the marker that showed statistical significant association between its genetic polymorphism and high risk TIA recurrence), it was found that elevated serum TNF-α level was associated with higher risk TIA recurrence. Similarly, high serum TNF-α level was linked to increased risk of ischemic stroke in the Chinese Han population (Cui et al., 2012). Not in favour of our results, the TNF-α was not connected to the risk of stroke in a British previous research (Jefferis et al., 2009). In addition, a meta-analysis also established that TNF-α was associated with a 1.6-fold higher in ischemic stroke risk in Asian ancestry but not on European descendent (Pereira et al., 2007).

Moreover, a statistical significant relation was found between serum TNF-α level and genotype of -308 AA. Coming with these data a weakly association between TNF-α level and different genotypes of -308 G/A. However, the probability that other functional SNP associate with the -308 G/A, may affect the regulation of the level of TNF-α or their interaction play a further role in another pathological processes cannot be excluded (Jefferis et al., 2009). Furthermore, it was mentioned that gene polymorphism in the promoter region (-308 G/A) could control and regulate the expression of serum TNF-α in another diseases such as rheumatoid arthritis (Mosaad et al., 2011).

Ideally, studies need several hundred patients to clarify major polymorphism departure that may play a major role in stroke and TIA development especially in young patients.

Many probable clarifications should be considered for this result. First, the identified TIA events not usually referred to neurology clinic. Second, the high risk group in the present study who had recurrent TIA assessed by non-clinical risk factors not ABCD2 score which may be insensitive. Third, we cannot exclude the possibility that our study is low power due to lack of short and long term follow up additionally small size number (Rothwell et al., 2005; Dennis et al., 1990).

In brief, the results of the current study obviously specify a positive association between TNF-α-308 G/A SNP and recurrent TIA. Nevertheless, these polymorphisms may confirm the prediction power of cytokines polymorphisms and recurrent TIA with subsequent high risk of actual stroke development and this manner may contribute to primary stroke prevention.

Acknowledgment
The authors would like to thank patients and their families for their patience and cooperation.

References
hypertension using the extreme discordant phenotype design. Hypertens Res; 32(9):775-9.


