Association of interleukin-10 gene promoter variants with ischemic stroke among the Pakistani population

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ABSTRACT

Genetic variations in Interleukin-10 (IL-10), an anti-inflammatory cytokine, has the potential to influence the development of Ischemic Stroke (IS). The current study aimed to examine IL-10 gene polymorphisms (rs1800896, rs1800871 and rs1800872) in association with IS. After genotyping, associational analyses showed a significant correlation of rs1800896 with the elevated risk of disease. Whereas for rs1800871, a decreased risk was observed and it might play a protective role. However, rs1800872 did not reveal an association with the predisposition to IS. Furthermore, all the haplotypes were significantly associated with the disease risk except GCA. The acquired D’ value from linkage disequilibrium showed the tendency of co-inheritance. Thus, the obtained results suggested that rs1800896 and rs1800871 have a significant relationship with IS.

Keywords: Ischemic stroke, IL-10, genotyping, polymorphism

INTRODUCTION

Ischemic Stroke (IS) is the second main cause of death and a major reason for disability globally and is considered the leading worldwide neurological problem [1]. World Health Organization (WHO) estimated that almost 17.9 million individuals deceased because of cardiovascular diseases (CVDs) worldwide. Among them, 85% of deaths were caused due to stroke and Coronary Heart Disease (CHD) [2]. IS is a multifactorial disease influenced by genetic
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and environmental factors and involves different pathophysiological pathways [3]. Various predisposing factors are the main causes for IS pathogenesis, such as gender, age, Diabetes Mellitus (DM), Hypertension (HTN), dyslipidemia, depression, physical inactivity, family history of the disease, tobacco consumption and genetic variations [4-6]. A number of genes have been involved in different signaling pathways, which may contribute to the pathogenesis of IS. Some of them are N-methylpurine DNA glycosylase (MPG), nitrogen permease regulator-like 3 (NPRL3) and interleukin-10 [6,7].

Interleukin-10, an anti-inflammatory and immunoregulatory cytokine, is primarily produced through immune cells like monocytes and lymphocytes [3]. IL-10 downregulates the inflammatory responses by preventing the formation of several pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) [8]. Furthermore, IL-10 prevents the production of nuclear factor-kappa B (NF-κB) and matrix-degrading metalloproteinases (MMPs). It also stimulates the T helper type 2 (Th2) expression and modulates the vascular inflammatory progresses and stability of plaque [9]. In IS, extreme inflammation may lead to thrombotic or atherosclerotic vascular lesions. Therefore, the IL-10 gene polymorphisms change the actual function of IL-10 and may impact IS development [10]. The current study aimed to examine the association between the Single Nucleotide Polymorphisms (SNPs) of the IL-10 gene (rs1800896, rs1800871 and rs1800872) with the susceptibility to IS in the targeted population. By exploring their role, these polymorphisms might be used as a potential biomarker for early detection of predisposition to IS.

MATERIALS AND METHODS

Ethical approvals and samples collection

To conduct the research, a total of 360 subjects were enrolled, which include 160 cases of IS patients and 200 healthy individuals as controls. The ratio of cases to controls was 4:5. Ethical approvals were taken from the Institutional Ethics Committee of the Karachi Institute of Biotechnology and Genetic Engineering (KIBGE) and Institutional Review Board of Jinnah Postgraduate Medical Center (JPMC), Karachi, Pakistan. The study protocols were under the ethical standards and guidelines of the 1975 Helsinki Declaration. The patients diagnosed through neuroimaging based on Computerized Tomography (CT) scan were recruited in the study, and patients bearing
malignancies, chronic infections and autoimmune disorders were excluded.

After getting the enrollees' informed consent from Jinnah Postgraduate Medical Center (JPMC), Karachi, Pakistan, a 5 ml blood sample in Ethylene Diamine Tetra-Acetic acid (EDTA) vacutainer was collected to prevent coagulation and kept at -20 °C till further process.

**Extraction of DNA and genotyping**

DNA extraction was performed through the salting-out method [11]. The DNA samples were quantified through a spectrophotometer (IMPLEN NanoPhotometer® P-Class, Germany). DNA integrity was determined through agarose gel (0.8 %) electrophoresis. Genotyping of SNPs was done through tetra primer ARMS-PCR using the thermal cycler (MOLEQUELE-ON, New Zealand). For this purpose, primer designing was carried out using an online available tool, Primer1.

A single reaction of 25 µL was prepared for PCR with 10 mM of each primer (MOLEQUELE-ON, New Zealand), 50 ng/µL DNA and 2x DreamTaq Green Master Mix (Thermo Scientific, Lithuania). For final volume makeup, Milli-Q water was used.

**Interleukin-10 gene promoter variants**

The PCR conditions of the targeted SNPs included initial denaturation (95 °C for 5 min) proceeded by denaturation (94 °C for 45 sec), annealing (58 °C for rs1800896, 56 °C for rs1800871 and 62 °C for 1800872, for 45 sec), extension (72 °C for 45 sec) and final extension (72 °C for 5 min) for 35 cycles. Separation of the amplified products was carried out on agarose gel (2.5 %) stained with VisualaNA (A) (MOLEQUELE-ON, New Zealand) against a 100 bp marker. The gels were visualized through a gel imaging system (FastGene®FAS-V, NIPPON Genetics Germany).

**Sequencing**

To validate the results of tetra primer ARMS-PCR, sequencing was performed. The polymorphic region of forward outer and reverse outer primers was amplified. The PCR product purification was carried out through a column purification kit (MOLEQUELE-ON, New Zealand). After sequencing, the data analysis was done by Molecular Evolutionary Genetics Analysis (MEGA 7.0).

**Statistical analysis**

IBM SPSS version 25 (IBM Corp., Armonk, NY, US) was used to analyze the obtained data. Chi-square (χ²) test was used to check the deviance from Hardy-
Weinberg Equilibrium (HWE) in the controls group and to compare the genotypic data of controls and IS cases. Association analyses were assessed through the Odd Ratio (OR) with a 95% Confidence Interval (CI). Moreover, the genetic models (co-dominant, dominant and recessive) were used to validate the association analyses. P-value ≤ 0.05 was considered as a significance level. Haplotype analyses and Linkage Disequilibrium (LD) between SNPs were estimated using the SHEsisPlus software.

RESULTS
Genotype distribution
Tetra primer ARMS-PCR analysis revealed that all the three polymorphisms rs1800896, rs1800871 and rs1800872 carried three forms of genotypes with different product sizes as shown in Figure 1. The sequencing results showed the representative electropherograms of genotypes in the samples as demonstrated in Figure 2. In the current study, rs1800896 and rs1800871 showed a significant difference between genotypic percentages of IS patients and controls (P < 0.001, χ² = 17.9) and (P < 0.01, χ² = 13.7), respectively while rs1800872 did not show any difference (P > 0.05, χ² = 1.02). HWE was tested in the controls group and the results revealed that rs1800896 and rs1800871 were consistent with HWE (P > 0.05) but rs1800872 was found to be inconsistent (P < 0.05). Furthermore, the OR revealed a significant correlation of polymorphism rs1800896 (A/G) with the elevated risk of IS (P < 0.05, OR = 1.55 [95% CI = 1.0-2.4]) while, rs1800871 (T/C) indicated a substantial decreased risk (P < 0.01, OR = 0.55 [95% CI = 0.3-0.8]). However, rs1800872 (A/C) was not significantly associated with the predisposition to IS (P > 0.05, OR = 1.15 [95% CI = 0.7-1.7]) as shown in Table 1. Moreover, for rs1800896, co-dominant (AG vs AA: P < 0.001, OR = 2.7 [95% CI = 1.7-4.2]) and dominant model (AG+GG vs. AA: P < 0.001, OR = 2.5 [95% CI = 1.6-3.9]) showed a significant association of the heterozygous AG genotype with the higher risk of IS. Whereas, under recessive model (GG vs. AA+AG: P > 0.05, OR = 0.9 [95% CI = 0.4-1.9]) rs1800896 was not found to be associated with the risk of disease as presented in Table 2. For rs1800871, co-dominant (CC vs. TT: P < 0.01, OR = 0.3 [95% CI = 0.1-0.6]), dominant (TC+CC vs. TT: P < 0.05, OR = 0.5 [95% CI = 0.2-0.9]) and recessive model (CC vs. TT+TC: P < 0.001, OR = 0.5 [95% CI = 0.3-0.7]), revealed that the variant CC genotype had
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a correlation with the decreased risk of the disease. However, 1800872 did not reveal any significant relationship with the susceptibility to IS under co-dominant (CC vs. AA: \( P > 0.05, \ OR = 1.4 \ [95\%\ CI = 0.7-2.9]\)) and (AC vs. AA: \( P > 0.05, \ OR = 1.3 \ [95\%\ CI = 0.6-2.5]\)), dominant (AC+CC vs. AA: \( P > 0.05, \ OR = 1.3 \ [95\%\ CI = 0.7-2.6]\)) and recessive models (CC vs. AA+AC: \( P > 0.05, \ OR = 1.2 \ [95\%\ CI = 0.7-1.8]\)) as demonstrated in Table 2.

**Figure 1.** Electrophoresis band pattern of the products of Tetra primer ARMS-PCR. (A) rs1800896. Lane 1: 100bp DNA Marker; Lanes 2, 5, 7 and 8: AA; Lanes 3, 6 and 9: AG; Lane 4: GG genotype. (B) rs1800871, Lane 1: 100bp DNA Marker; Lanes 2 and 9: TT; Lanes 3, 4, 6, 7, 8, 10, 11 and 12: TC; Lane 5: CC genotype. (C) rs1800872, Lane 1: 100bp Marker; Lanes 2,3,5,10,11 and 12: AC; Lanes 4, 7, 8 and 9: CC; Lane 6 and 13: AA genotypes.
**Figure 2.** Electropherogram of sequenced DNA samples of *IL-10* gene polymorphisms. (A) AA, GG and AG genotypes of rs1800896. (B) TT and TC genotypes of rs1800871 (C) AA, AC and CC genotypes of rs1800872.
Table 1. Distribution and association of *IL-10* gene polymorphisms with the susceptibility to IS

<table>
<thead>
<tr>
<th><em>IL-10</em> gene</th>
<th>Genotypic Percentages</th>
<th>Allelic Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotypic Percentages</td>
<td>Allelic Frequencies</td>
</tr>
<tr>
<td>rs1800896</td>
<td>AA  94(47) 87(44) 19(9)</td>
<td>17.9 0.0001 0.861</td>
</tr>
<tr>
<td></td>
<td>GG  0.69/0.31</td>
<td>HWE (P-value)</td>
</tr>
<tr>
<td></td>
<td>Controls n(%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cases n(%)</td>
<td></td>
</tr>
<tr>
<td>rs1800871</td>
<td>TT  14(7) 80(40) 106(53)</td>
<td>13.7 0.001 0.835</td>
</tr>
<tr>
<td></td>
<td>CT  0.55 (0.3-0.8)</td>
<td>Odds Ratio (95% CI)</td>
</tr>
<tr>
<td></td>
<td>CC  0.0094</td>
<td>P-value</td>
</tr>
<tr>
<td>rs1800872</td>
<td>AA  26(13) 111(55) 63(32)</td>
<td>1.02 0.601 0.034</td>
</tr>
<tr>
<td></td>
<td>AC  0.41/0.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC  1.15 (0.7-1.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls n(%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cases n(%)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Linkage disequilibrium patterns among rs1800896, rs1800871 and rs1800872 polymorphisms of *IL-10* gene.
Table 2. Genetic model association of *IL-10* gene polymorphisms with IS risk

<table>
<thead>
<tr>
<th>Genetic Models</th>
<th>rs1800896</th>
<th>Odds Ratio (95% CI)</th>
<th>rs1800871</th>
<th>Odds Ratio (95% CI)</th>
<th>rs1800872</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-dominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1.0</td>
<td>TT</td>
<td>1.0</td>
<td></td>
<td>AA</td>
<td>1.0</td>
</tr>
<tr>
<td>AG</td>
<td>2.7 (1.7-4.2)</td>
<td>TC</td>
<td>0.6 (0.3-1.4)</td>
<td></td>
<td>AC</td>
<td>1.3 (0.6-2.5)</td>
</tr>
<tr>
<td>GG</td>
<td>1.6 (0.7-3.6)</td>
<td>CC</td>
<td>0.3 (0.1-0.6)</td>
<td></td>
<td>CC</td>
<td>1.4 (0.7-2.9)</td>
</tr>
<tr>
<td>Dominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1.0</td>
<td>TT</td>
<td>1.0</td>
<td></td>
<td>AA</td>
<td>1.0</td>
</tr>
<tr>
<td>AG+GG</td>
<td>2.5 (1.6-3.9)</td>
<td>TC+CC</td>
<td>0.5 (0.2-0.9)</td>
<td></td>
<td>AC+CC</td>
<td>1.3 (0.7-2.6)</td>
</tr>
<tr>
<td>Recessive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA+AG</td>
<td>1.0</td>
<td>TT+TC</td>
<td>1.0</td>
<td></td>
<td>AA+AC</td>
<td>1.0</td>
</tr>
<tr>
<td>GG</td>
<td>0.9 (0.4-1.9)</td>
<td>CC</td>
<td>0.5 (0.3-0.7)</td>
<td></td>
<td>CC</td>
<td>1.2 (0.7-1.8)</td>
</tr>
</tbody>
</table>
Table 3. Association between haplotypes of rs1800896, rs1800871 and rs1800872 with the risk of IS.

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Cases (Frequency)</th>
<th>Controls (Frequency)</th>
<th>$\chi^2$</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-C-C</td>
<td>49(0.153)</td>
<td>110(0.275)</td>
<td>15.346</td>
<td>&lt;0.001</td>
<td>0.48 (0.33-0.69)</td>
</tr>
<tr>
<td>A-T-C</td>
<td>44(0.137)</td>
<td>31(0.077)</td>
<td>6.858</td>
<td>0.008</td>
<td>1.89 (1.17-3.08)</td>
</tr>
<tr>
<td>A-T-A</td>
<td>69(0.215)</td>
<td>62(0.155)</td>
<td>4.389</td>
<td>0.036</td>
<td>1.49 (1.02-2.19)</td>
</tr>
<tr>
<td>G-C-C</td>
<td>102(0.318)</td>
<td>92(0.23)</td>
<td>7.113</td>
<td>0.007</td>
<td>1.57 (1.12-2.18)</td>
</tr>
<tr>
<td>A-C-A</td>
<td>26(0.081)</td>
<td>72(0.18)</td>
<td>14.743</td>
<td>&lt;0.001</td>
<td>0.40 (0.25-0.65)</td>
</tr>
<tr>
<td>G-C-A</td>
<td>15(0.046)</td>
<td>18(0.045)</td>
<td>0.014</td>
<td>0.904</td>
<td>1.04 (0.52-2.10)</td>
</tr>
</tbody>
</table>

Haplotypes frequency of < 0.03 was ignored.
Polymorphisms order; rs1800896, rs1800871 and rs1800872.

Linkage disequilibrium of IL-10 gene polymorphisms

The patterns of linkage disequilibrium among rs1800896, 1800871 and rs1800872 were analyzed. The correlations among polymorphisms were measured as $D'$ (Figure.3). The obtained $D'$ values of rs1800896 vs. rs1800871 were 0.64, 1800871 vs. 1800872 was 0.41, and for 1800896 vs. rs1800872, the $D'$ value was observed to be 0.52, which shows that these SNPs were in linkage disequilibrium.

Haplotypic association with IS risk

The haplotype analysis showed the combined effects of the three targeted SNPs. The ACC ($P < 0.001$, OR = 0.48 [95 % CI = 0.33-0.69]) and ACA ($P < 0.001$, OR = 0.40 [95 % CI = 0.25-0.65]) haplotypes showed a significantly decreased risk of disease while ATC ($P = 0.008$, OR = 1.89 [95 % CI = 1.17-3.08]), ATA ($P = 0.036$, OR = 1.49 [95 % CI =
Ischemic stroke is the second major cause of morbidity and the common reason for mortality across the globe [1]. Stroke is particularly common among the population of South Asia due to the burden of many risk factors, such as positive family history of IS, HTN, smoking, dyslipidemia, sedentary lifestyle and genetic variations [6,12,13].

Genetic alterations in the *IL-10* gene play an essential role in the development of different inflammatory diseases, including stroke [14]. Polymorphisms in the *IL-10* gene are the promising risk factors of IS. Lower production of IL-10 in an individual may lead to the progression of vascular diseases [15]. However, under common physiological conditions, expression of IL-10 may sustain the stability between pro-and anti-inflammatory cytokines [3].

It has been estimated that approximately 75% of the IL-10 production level is determined genetically [16]. It has been reported that polymorphisms in the *IL-10* gene have an inclination towards IS risk among Asians. However, inconsistent results have been obtained by various reported association studies [3,6,15]. Hence, the *IL-10* gene is considered as the candidate gene for cerebrovascular diseases. Furthermore, IL-10 has the ability to provide a neuroprotective effect through transcription-independent modulation of ischemia-induced intracellular Ca\(^{2+}\) reactions. Consequently, the *IL-10* gene expression is linked with the IS pathological mechanisms [17].

In the current study, it was observed that the AG genotype and A allele of rs1800896 showed a significant association with susceptibility to IS in the targeted population. It might be due to the A/G substitution of promoter region polymorphism (rs1800896) which may be associated with differential IL-10 production [18]. Additionally, under dominant and co-dominant models, the AG genotype was also significantly associated with the disease’s increased risk. However,
the frequency of variant GG genotypes was lower as compared to AA and AG genotypes. Similar findings were observed in Chinese populations, but in European populations, divergent results were reported [19,20]. A meta-analysis also reported the similar inconsistent findings [21]. Erratic findings among various studies might be due to different ethnicities, diversified genetic origins, socioeconomic status, sample size and other confounding factors. Furthermore, multiple genetic models revealed that CC and TC genotypes and the C allele of rs1800871 were associated with low risk, and it might play a protective role against IS. Similar outcomes were also observed in the Chinese population [6]. However, the polymorphism rs1800872 did not show any difference in genotypic distribution and correlation with the predisposition to IS. A recent meta-analysis also reported the same results [22]. Moreover, it was found that the haplotypes of the studied IL-10 gene variants were also significantly associated with the disease risk. LD was also observed between all the three SNPs, which shows the tendency of co-inheritance. Further studies with larger sample size may shed more light on the potential role of these SNPs with an increased predisposition to IS.

CONCLUSION

In conclusion, the obtained findings suggested that the SNPs rs1800896 and rs1800871 were associated with the susceptibility to IS and may play a potential role in clinical practice. Therefore, these SNPs may be used as a genetic biomarker for early detection of the disease to identify those individuals who are at a higher risk of developing IS. However, for a better understanding of the disease and to further confirm the results of the current study, more research will be required with the larger sample size and diverse ethnic groups.

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