CCL2 Polymorphism in Drug-Resistant and Drug-Responsive Patients with Epilepsy in Isfahan, Iran

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ABSTRACT

Background and objective: Approximately 50 million people worldwide (1% of the world's population) suffer from epilepsy. Among 700 thousand people with epilepsy in Iran, 20% have refractory epilepsy. Accumulation of leukocytes in patients' brain parenchyma is thought to be related to different types of epilepsy. Recent clinical observations suggest that therapeutic strategies that interfere with leukocytes or cause them to migrate may have therapeutic efficacy in epilepsy. The aim of this study was to identify treatment-resistant patients, and investigate the association between polymorphism rs1024611 in *CCL2* gene and drug resistance in patients with epilepsy in Isfahan, Iran.

Methods: Blood samples were taken from 50 patients with intractable epilepsy (case group) and 50 drug-responsive patients with epilepsy (control group). Genomic DNA was extracted from peripheral blood by salting out method. Specific primers were designed by Oligo 7 software to investigate polymorphism rs1024611 using PCR-RFLP. The preliminary results for a number of samples were confirmed by sequencing.

Results: The results of this study showed that there was a significant relationship between intractable epilepsy and presence of C allele.

Conclusion: Similar to previous study, we found a significant association between *CCL2* gene polymorphism and drug-resistant epilepsy.

Keywords: Epilepsy, Drug Resistance, Polymorphism, CCL2.

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INTRODUCTION

Approximately 50 million people worldwide suffer from epilepsy, and almost 80% of cases occur in developing countries (1). In Iran, 700 thousand people have been diagnosed with epilepsy. While 80% of the patients respond to drug therapy, 20% of the patients have refractory epilepsy (do not respond to therapy) (2). Recently, it has been found that leukocytes play a key role in the pathogenesis of epilepsy, and inhibition of leukocyte surface receptors has anti-therapeutic effects. Studies show that leukocytes' accumulation in brain parenchyma of patients is associated with different types of epilepsy. Recent clinical observations suggest that interference with or migration of leukocytes may have therapeutic effects on epilepsy. Evidence suggests that the presence of inflammatory mediators, such as interleukin-1, toll-like receptors, high-mobility group box protein 1 and chemokine C-C motif ligand 2 (CCL2) has a major role in the development of intractable epilepsy. It was observed that inflammatory pathway genes are involved in intractable epilepsy in both animals and humans. Chemokine CCL2 is associated with inflammatory diseases, migration of inflammatory cells and targeting of cells (3). CCL2 expression regulates epilepsy status in animals and patients with refractory epilepsy. Previous studies have shown that injection of pilocarpine (a drug used for treatment of dry mouth and glaucoma) before epilepsy will alter the expression of CCL2 (3). Recently, an integrative analysis of large-scale gene expression in the brain tissue have shown the different expression pattern of genes involved in neuroinflammation (4), including CCL2 that has had different and fixed expression. Investigation of the role of leukocytes and their adhesion mechanism in seizure could help develop novel therapeutic approaches for epilepsy (4). Because of the higher risk of mortality in treatment-resistant patients, it is essential to seek alternative treatment options (4). The aim of this study was to identify treatment-resistant patients, and investigate the association between polymorphism rs1024611 in *CCL2* gene and drug resistance in patients with epilepsy in Isfahan, Iran.

Gene position

CCL2 gene is located on human chromosome 17 (q11.2) and has 3 exons and 99 amino acids (5). The gene is one of the several cytokine gene clusters on the q-arm of chromosome 17. Chemokines are a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes (6). CCL2 is a member of the C-C chemokine family, which is characterized by two adjacent cysteine residues. The superfamily is divided into four subfamilies based on the arrangement of N-terminal cysteine residues of the mature peptide. The *CCL2* gene plays a key role in disruption of the blood-brain barrier (BBB) integrity. Overexpression of CCL along with other inflammatory mediators leads to dysfunction of monocytes and lymphocytes during epilepsy activity, which impairs the integrity of the BBB (7).

MATERIAL AND METHODS

This case-control study was performed on 100 patients (55 women and 45 men) with epilepsy who were referred to the Epilepsy Charity Center, in Isfahan. After obtaining consent from participants, blood samples were collected. Necessary measures were taken by neurologists at the Epilepsy Center for the selection of the study group. The mean age of patients was 28 years (age range: 6-50 years). Based on the subjects' response to therapy, they were divided into two groups of drug-resistant (have used more than two drugs for a year, but the seizure and the frequency of seizures were not reduced) and drug-sensitive (50 subjects each).

Blood samples were transferred to laboratory on an ice bucket, and then stored at -20 °C. Genomic DNA was extracted from the blood samples by salting out as described by Miller et al. (8). To determine genotype of the *CCL2* gene by polymerase reaction process (PCR), two primers were designed using Oligo7 software for *CCL2* rs1024611 polymorphism. The PCR reaction solution contained 19 µl water, 2.5 µl buffer (CinnaGen), 0.75 µl $MgCl_2$ (CinnaGen), 0.5 µl dNTP (CinnaGen), 0.75 µl of each primer (Bioron Co.), 1.5 µl DNA, and 0.3 µl DNA polymerase (CinnaGen). Amplification process for the sequence was done in a thermocycler (Bio-Rad, Germany) at annealing temperature of 56 °C in 35 cycles. Restriction enzyme with at least one position for the desired sequence was prepared using SGD website for performing restriction fragment length polymorphism

(RFLP) analysis for the desired sequence. Restriction enzyme *PUVII* (Fermentase) recognizes CAG^CTG sites and cuts best at 37 ^{o}C (Figure1). PCR products were electrophoresed on 3% agarose gel stained with ethidium bromide, and then observed under UV light. Moreover, some of the products were sequenced (Macrogen Inc., South Korea) to ensure the accuracy of the results obtained by RFLP. Data was analyzed using SPSS software and one-way analysis of variance (ANOVA). P-values less than 0.05 were considered as statistically significant. Tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR) was available for detection of polymorphisms 1024611.

RESULTS

In this study, we investigated the rs1024611 polymorphism in the *CCL2* gene among 50 drug-resistant epilepsy patients and 50 drug responsive epilepsy patients (as controls). No significant association was found in terms ofage, gender, and history of neurological diseases. The genotype and allele frequency distributions in drug-responsive and drug-resistant patients with epilepsy are shown in Table 1. Evaluation of genotype distribution showed that TT frequency is higher in the case group compared with the control group, but this difference was not statistically significant. As shown in Table 2, frequency of TC genotype was 40.3% and 41.9% in the case group and control group, respectively (P=0.171). Frequency of CC was 8.4% in the case group and 6.5% in the control (P=0.033). There was a significant relationship between the frequency of allele C and intractable epilepsy (P=0.026). Some of the PCR products were sequenced to ensure accuracy of the results obtained (Figure 2).

Table 1- Genotype distribution of CCL2 among the two study groups

Variables		Number	337(+)	234	df	sig
Age	6-15	15	15	15	17	.048
	16-30	35	35	35	78	.051
	31-50	40	40	40	68	.669
Gender	Female	55	48	48	114	.085
	Male	35	42	42	96	.361
Family relations	Related	24	24	12	85	005.
	Not-related	66	66	26	88	.250
History of neurological disease	Yes	30	30	9	56	045.
	No	60	60	22	112	321.
Responding to treatment	-	59	59	38	713	0.00
Not responding to treatment	-	31	31	52	711	025.

Table 2- Frequency of alleles among drug-resistant and drug-responsive patients with epilepsy

Genotype/allele	Drug-resistant N (%)	Drug-responsive N (%)	OR (95% CI)	P value
TT	34(54.9%)	16(51.6%)	Reference	
TC	25(40.3%)	13(41.9%)	1.90(0.307-2.216)	0.171
CC	3(8.4%)	2(6.5%)	2.70(0.107-4.651)	0.033
TC+CC	28(51.7%)	15(48.4%)	1.878(0.070-2.084)	0.070
T	93(75%)	45(72.6%)	Reference	
C	31(25%)	17(27.4%)	1.88(1.04-1.76)	0.026

Figure 1- Results of electrophoresis on PCR products obtained by *PVUII* enzymatic digestion. Column1: uncut PCR product, Column 2: TC heterozygotes, Column 3: TT homozygous, Column 4: TC heterozygotes, Column 5: 100bp DNA ladder, Column 6: TC heterozygous, Column 7: TT homozygous, Column 8: TT homozygous, Column 9: TT homozygous.

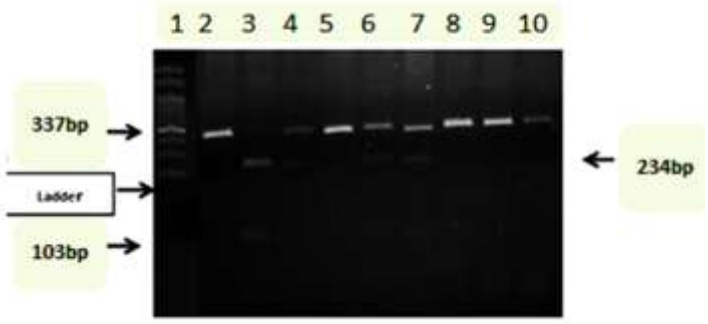
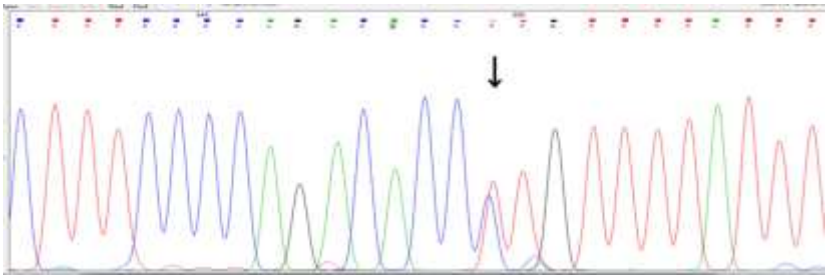


Figure 2- Results of sequencing of TC *CCL2* rs1024611 genotype



DISCUSSION

Approximately 7-8% of people worldwide have experienced a seizure some time during their lifetime, which it is often accompanied by fever. Among the 700 thousand people suffering from epilepsy in Iran, about 200 thousand patients have intractable epilepsy, and do not respond to treatment (3). Evidence suggests that some chemokines play a proinflammatory role during inflammation, and attract immune cells to the infection site. Recently, it has been found that leukocytes have a major role in the pathogenesis of epilepsy (9). Studies show that leukocytes' accumulation in brain parenchyma of patients is associated with different types of epilepsy (4). Recent clinical observations suggest that interference with or migration of leukocytes may have therapeutic effects on epilepsy (4). Investigation of the role of leukocytes and their adhesion mechanism in seizure could help develop novel therapeutic approaches for epilepsy (4). Despite the introduction of new antiepileptic drugs, 20-30% of patients with epilepsy do not respond to medication (5). It was observed that inflammatory pathway genes play an important role in intractable epilepsy in both animals and humans (5). CCR2 and CCL2 expression has been observed in cells of various brain regions. The *CCL2* gene is involved in a number of diseases and nervous system inflammatory disorders. Studies show that rs1024611 polymorphism in the *CCL2* gene can affect its expression (9). Rovinj et al. have shown that polymorphisms found in peripheral blood cells affect expression of MPC-1 in response to inflammatory stimuli (10). In addition, bell-like protein has been found effective in reduction of seizures (5). Study of Arisi et al. have shown that the injection of pilocarpine before epilepsy alters the expression of CCL2 (11). Studying the relationship between rs1024611 polymorphism and CCL2 expression level has shown that they have different haplotypes, and outcome of the *CCL2* gene polymorphisms may be very complex (12). The genes involved in intractable epilepsy have been identified by microarray study of patients with the disease (6). Consistent with our findings, Lee et al. found an association between intractable epilepsy and *CCL2* rs1024611 polymorphism in children with intractable epilepsy (4).

CONCLUSION

The results of this study show that there is a significant relationship between *CCL2* rs1024611 polymorphism and intractable epilepsy.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

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