Studying mutation in GABRG2 Gene involved in occurrence of grand Mal epilepsy among Iranian patients using PCR-SCCP/HA methodology

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ABSTRACT

Epilepsy is one of the most common complications of the central nervous system with the highest frequency in human communities following heart failures and brain stoppage. A research conducted on families suffering from epilepsy revealed both single-gene and multi-factor epilepsy. There are many genes that cause epilepsy. One of the major causes of epilepsy is mutation of the genes encoding various types of ion channels units. The present research seeks to study the role of structural mutations in GABRG2 gene in causing epilepsy with positive family background or lack of any caused for those suffering from epilepsy. Through convenient sampling, the blood samples of 60 patients suffering from tonic-clonic grand mal epilepsy were collected. The type of epilepsy was determined based on what was in their medical file and record. Then, the full DNA of white globules was extracted using phenol-chloroform standard, and using the primers designed for exons 8, 9, and 10 of GABRG2 gene, polymerase chain reactions (PCR) were carried out using the standard method. PCR products were analyzed using SSCP, Chromas, BLASTn servers and ClustalW. Pointwise structural mutations were observed in two samples. Both mutations recognized in this gene are new. In this research, a missense mutation (c.914 > G) was diagnosed in GABRG2 gene which turned Adenine into Guanine in 914- nucleotide situation. Furthermore, a missense mutation was observed in the nucleotide situation 1186. This mutation changed thymine to guanine (c.1186T>G) and transformed leucine amino acid into Valine 396. The present research describes the correlation of GABRG2 gene mutation with IGE. Generally speaking, based upon the current research and the previous researches conducted by the authors, GABRG2 gen can be used a diagonal marker for idiopathic epilepsies.

Keywords: Epilepsy, GABRG2 Gene, Mutation, PCR-SSCP.
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INTRODUCTION

Epilepsy is one of the most common complications of the central nervous system and the patient suffering from it experiences seizure and convulsion at least twice. This disease is diagnosed through repetitive convulsions in the brain caused by the excessive stimulation of the neurons. Epilepsy does not just affect the patient; it has various effects and influences on the family of the patient, too. Nearly 65 million people are suffering from epilepsy in the whole world now. This disease carries with it a big burden of disabilities associated with epilepsy, death, comorbidities and costs families a lot of money [1, 2]. Nearly 700 thousand people are suffering from epilepsy in Iran which is a very important economic and social challenge [3]. Mutation of GABGR2 that encodes GABA S receiver $\gamma2$ sub-unit can be the cause of both genetic epilepsy (GEFS) and Dravet syndrome [4]. Patients with Dravet syndrome or severe myoclonic epilepsy in infancy (SMEI) suffer from various treatment-resistant convulsions over the first year and in the following years, the usually suffer from unusual convulsions, myoclonus

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and mild convulsion [5]. GEFS[+] is a dominant autosomal channelopathy disorder which can influence many family members or turn from automatic mutations into a cause of epilepsy [6].

GABA or gamma-aminobutyric acid is one of the most important inhibitory neurotransmitters in our central nervous system. GABA neurotransmitter is produced through a specific metabolic path titled GABA shunt from glutamic acid. There are three receptors (GABA$_A$, GABA$_B$, and GABA$_c$) for GABA molecule. GABA$_A$ and GABA$_B$ receptors are ionotropic neurotransmitting receptors that act in the form of valvate ion channels dependent upon ligand. On the other hand, GABA$_B$ receptors belong to the metabotropic family and the receptors pairing with –G proteins [7]. All the mutations reported were observed in GABA A receptor [8]. GABRG2, GABRA1, and GABRD are some of the genes encoding GABA A receptor. γ2 sub-unit receptor of GABA is encoded by GABRG2 gene. This gene is situated in situation 5q34 and its frequency in the brain is high [9]. The receptors utilized included the 5 sub-units selected from 19 known receiving sub-units α1-6, β1-3, γ1-3, $\delta, \varepsilon, \theta, \pi$ and ρ1-3. This random assembly is the result of a complicated heterogeneity of GABA A receiving cub-types [10]. All the sub-units receiving GABA A possess an big N-terminal extracellular domain containing cysteine loop, 4 heavily protected transmembrane domains (TMS) and an intracellular loop between TM3 and TM4 [11]. Each sub-unit coded by GABRG2 gene contains a ligand binding domain (LBD) to the big N-terminal extracellular domain, 4 transmembrane spirals (TM1-4) and a big intercellular loop between TM3 and TM4 [12]. Two heterozygous missense mutations from GABRG2, P83S, and N79S were reported in the other forms of idiopathic and genetic general epilepsies [13, 14].

GEFS[+] is correlated with missense and senseless mutations in GABRG2 (Q40X, K328M, Q390X, R136X, Y444Mfs51X, W429X) [6, 15, 16]. Dravet syndrome is now supposed to be caused by two heterozygous senseless mutations of Q40X and Q390X [16].

In this research, GABRG2 gene is discussed as candidate gene that causes unknown epilepsies with positive family background. Considering the great frequency of epilepsy in Iran (nearly 700 thousand people) and the importance of economic and social consequences of this disease, we decided to investigate the possible genetic changes of GABRG2 gene among the Iranian patients suffering from idiopathic epilepsy.

Our research is based on this hypothesis: mutation in the encoding gene of γ2 subunit from GABA A receptor is correlated with occurrence of epilepsy in Iran.

MATERIALS AND METHODS

Patients and samples
Epilepsy includes a vast range of clinical demonstrations. Due to great variance of epilepsy while selecting the patients, it is not possible all cases of general idiopathic epilepsy. Thus, we restricted our research to the patients suffering from general grand mal epilepsy with toni-clonic attacks.

The current descriptive-laboratory research was conducted in the period of January 2013 to January 2015. The blood samples of the patients were collected through convenient sampling method after gaining their written formal consent in order to gain access to their medical files. The research was approved by the ethics committee of the hospital. Phenotypes were categorized based upon commission and the terms of international anti-epilepsy league (1981), and the epileptic syndromes were also registered with reference to the current versions of this commission [17, 18, 19, 20]. The present research was conducted by studying the group including 60 patients suffering from general grand mal epilepsy along with tonic – clonic attacks based upon the diagonal criteria of international anti-epilepsy institution. In this step, 5 ml arterial blood was taken from patients and stored in tubes containing EDTA.

Extracting DNA and conducting PCR
Blood samples from 60 people icluding 30 men and 30 women aging from 10 to 30 years old were collected. The standard phenol-chloroform method was utilized to extract DNA (21). To design the primers, first the sequence correlated with the gene encoding γ2 sub-unit (GABRG2) from GABA A receptor with the access number NG_009290,1 was received from Ensembl database. For more validity and certainty, the above-said sequence was compared with the sequence of this gene in NCBI database and it turned out that both were the same. Then, the primers were designed using GeneRunner 3.0 software for exons 8, 9, and 11 of GABRG2 gene starting from a bit above the main range of our exon. It was then sent over to Sina Clone Company for synthesis.

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Table 1: the starters used to conduct PCR

Sequence of primet (5 → 3)	Melting point °C	Length	Exon	Primer's name
F: CAGTGATTGATAAAGGGTTG R: TGAGAAACTGCAACATAAAC	52	232	8	G3
F: TCCTGACAATGACCACCC R: TGCCAATGGAAAATGATAC	50	217	9	G1
F: CTCGTCCCAGGCCCCTAC R: GATACATACAGGGTTTTTCTTC	55	221	9	G8
F: CTCGTCCCAGGCCCCTAC R: AACCCATACCTCCTCACAG	55	299	11	G9

The conditions of proliferation through PCR reaction are available if demanded. PCR products were run on 1% agars gel using TBE 1x. They were observed under ultraviolet ray. To determine DNA sequence, PCR products were sent over to Pishgam biotechnology company and Chromas software was used to analyze the results.

SSCP and determining the sequence
Having conducted the usual PCR for genome DNA with high distinguishing power, SSCP reaction and determination of the standard unidirectional automatic variation of DNA from exons and proliferating areas of exon-intron was achieved using ABI 3700 capillary technology (Gen Fan Avaran, Macrogen, South Korea). All products of PCR were analyzed using SSCP. For this purpose, 8 ml of each PCR product was loaded with 6 ml buffer and their contents are displayed in table 2.

Table 2: Materials and the volume of each one needed to produce SSCP mixture

Material	Amount
Formamide	3 ml
Loading buffer	2 ml
0.1 molar soda	1 ml
PCR product	8 ml

After denaturation in a temperature of 98 °C for 10 minutes, the samples were immediately put in ice and, then, loaded in10% Acrylamide gels: Acrylamide-bis (1:29) in TBE 1x buffer and a voltage of 150 volts for 14 hours after a 250 volts pre-execution for 5 minutes. PCR products who displayed different electrophoretic patterns by PCR-SSCP/HA were sequenced in a unidirectional fashion using ABI machine. Pieces of DNA were finally revealed using silver staining method.

RESULTS

In the sample where exon number 8 is engaged, the corresponding change has taken place in the second trans-membrane are (TM2). This change takes place replacing the nucleotide in position 914 of exon 8 of nucleotide sequence (c.914A>G). This new codon (TCG) is represented in exon sequence of GABRG2 gene (Figure 1).
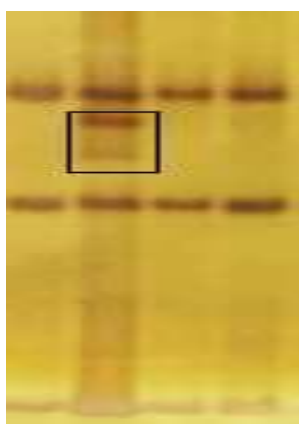
Figure 1: SSCP pattern of proband for exon 8 in GABRG2. SSCP analysis showed a new conformer in denatured product of patient. The samples are run in 10% acrylamide gel

Sequencing the reverse thread of this piece, showed an exon mutation c.914A>g which caused the replacement of p.Y305C (figure 2).
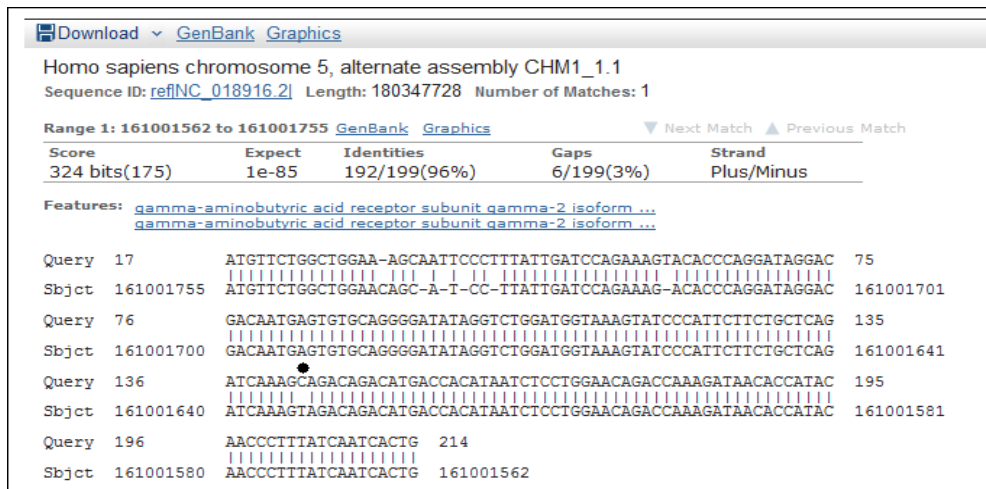

Figure 2: nucleotide blast between the normal and mutated GABRG2

Such changes in the second trans-membrane region of GABA A receptor plays a major role in formation of the chloride channel. The similarity observed between the sequence and nicotinic acetylcholine receptor predicted that ionotropic GABA receptors had for trans-membrane areas and TM2 formed the chloride channel [21, 22]. Therefore, such change can disrupt formation of channel wall cause abnormalities.
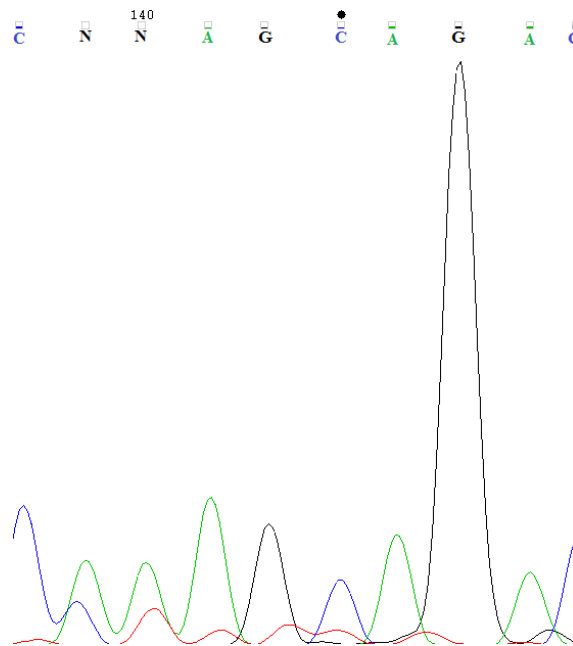

Figure 3: A914G mutation Chromatogram. Mutated nucleotide sequence is represented by a black dot in reverse string

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Horse    -GYPREEIVYQWKRSSVEVGDTRSWRLYQFSFVGLRNTTEVVKTTSGDYV
Human    DGYPREEIVYQWKRSSVEVGDTRSWRLYQFSFVGLRNTTEVVKTTSGDYV
Mouse    -GYPREEIVYQWKRSSVEVGDTRSWRLYQFSFVGLRNTTEVVKTTSGDYV
Pig      -GYPREEIVYQWKRSSVEVGDTRSWRLYQFSFVGLRNTTEVVKTTSGDYV
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Horse    VMTVYFDLSRRMGYFTIQTYIPCTLIVVLSWVSFWINKDAVPARTSLGIT
Human    VMSVYFDLSRRMGYFTIQTYIPCTLIVVLSWVSFWINKDAVPARTSLGIT
Mouse    VMSVYFDLSRRMGYFTIQTYIPCTLIVVLSWVSFWINKDAVPARTSLGIT
Pig      VMTVYFDLSRRMGYFTIQTYIPCTLIVVLSWVSFWINKDAVPARTSLGIT
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Figure 4: alignment of some sequences from a portion of human GABRG2 and correlated peptides and GABRG2 homologs of vertebrates with focus on the areas around 914 situation of human GABRG2 gene. The residue of situation 914 shows human γ2 sub-unit from GABA A (GABRG2) receptor

The other fact observed in the study of exon 9 showed that missense mutation results in transforming Thymine nucleotide into guanine in nucleotide situation 1186. As a result, transformation of leucine 396 amino acid into valine takes place in the intercellular loop.
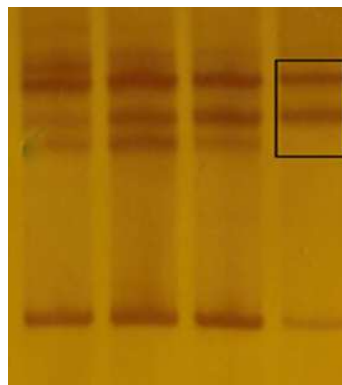

Figure 5: SSCP pattern of proband for exon 9 in GABRG2. SSCP analysis revealed a new conformer in denatured product of the patient. The samples were run in 10% acrylamide gel

Sequencing the go string of this piece showed a c.1186t>G exon gene which results in replacement of p.L396V (figure 6).
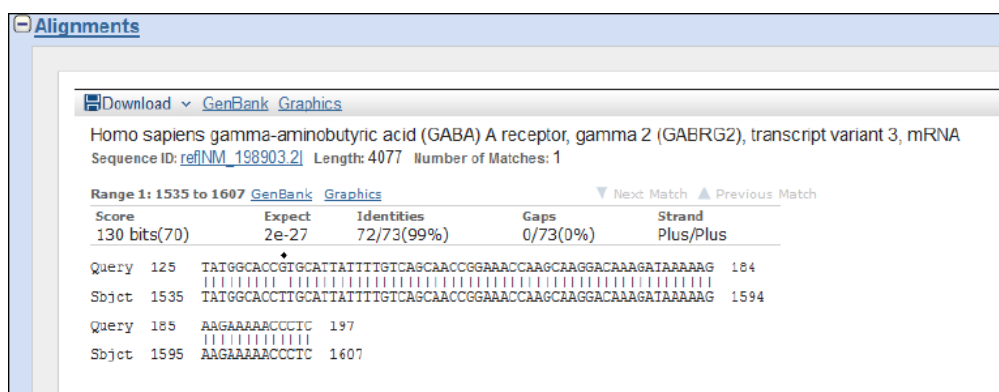

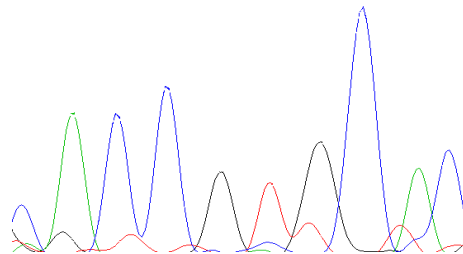**Figure 6: nucleotide blast of GABRG2 between mutated and normal sequences**

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Figure 7: T1186G mutation chromatogram. The mutated nucleotide sequence is represented by a black dot on the "go string"

Figure 8. Alignment of some sequences of a portion of human GABRG2 with associated peptides and GABRG2 homologs of vertebrates with a focus on the areas around situation 1186 of human GABRG2 gene. The residue of situation 1186 shows human γ2 sub-unit from GABA A (GABRG2) receptor

DISCUSSION

Epilepsy is one of the most common disorders of the central nervous system and genes play a major role in its occurrence. One of the genetic changes that causes this disease is mutation of GABRG2 gene. The present paper studied the screening of GABRG2 gene mutations in three gene exons whose clinical importance has already been proved. The patients were selected randomly in Tehran province. The contents of their medical files and the ideas of doctors and specialists about the type of epilepsy (our case dealt with general epilepsy) were taken into consideration in the process of selecting them. Having repeated SSCP, some samples were sent to Pishgam Company to determine their sequence. These samples were correlated with exons 8, 9, and 11. Having compared the results of their chromatogram with the sequence in NCBI gene bank, two cases nucleotide differences were observed. The mutations observed in this research justify their roles in occurrence of epilepsy. The site of the corresponding amino acids in protein indicated that their sequence was protected among various species and human. In the sample where exon number 8 was involved, we observe a nucleotide change by turning nucleotide A to nucleotide G in situation 914. As a result, tyrosine amino acid in situation 305 turned into cysteine. This change takes place in the second trans-membrane area (TM2). TM2 area is composed of 5 sub-units next to one another and forms the wall of GABA A receptor's channel. As a result, the above said changes disrupt formation of the wall of receptor's channel and cause abnormalities as a result. Meanwhile, a missense mutation was reported in exon 9 of this gene and this mutation results in changing nucleotide T to its G counterpart in 1186 nucleotide position. For this

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purpose, Lucien 396 amino acid transformation into valine takes place in the intercellular ring of GABRG2 gene. This ring connects the proteins that play a role in locating GABA A receptor in Synapses and help it function correctly. For this purpose, this mutation can influence the natural performance of corresponding protein and the location where the receptor is placed [22].

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Table 3: the mutations reported in this research

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Exon studied	Nucleotide location of mutation	Nucleotide transformation	Normal amino acid of a normal person	Transformed amino acid
Exon 8	914	TAC ➔ TGC	Tyrosine	Cysteine
Exon 9	1186	TTG ➔ GTG	Lucien	Valine

In the research conducted by Wallace et al, R82Q missense mutation was for the first time observed in a big family including several people afflicted with idiopathic epilepsy. Out of 192 people studied in this research, 29 exhibited the above-mentioned mutation and had phenotypes of childhood absence epilepsy (CAE) and the febrile seizures (FS). This missense mutation was reported in N-terminal of GABRG2 gene [23].

As Crunelli at al have reported, the inappropriate oligomerization of sub-units will reduce the number of GABA receptors and sites of connection or Benzodiazepines and increase the level of excitability in human body [24].

Baulac et al reported another missense mutation in GABRG2 gene which is K328M. This research was conducted on a big French family suffering from idiopathic epilepsy observed after determining exon 8 sequence in GABRG2 gene [14].

In another research conducted by Ishee et al on a Japanese family with 2 dizygotic twin girls suffering from Dravet syndrome, Q40X mutation was reported. Johnston et al (2013) reported R136X missense mutation in a family suffering from FS. Bothe Q40X and R136X mutations had taken place in the 2nd exon of GABRG2 exon. NMD regulatory system was activated by forming early termination codon [5].

Concerning GABRG2 gene mutation, a research was conducted by Ahadi et al. using PCR-SSCP/HA technique and Sanger sequencing in Iran where 46 patients suffering from FS were studied. R82Q mutation was once again confirmed in a 12-year-old Iranian boy after checking the second exon of GABRG2 gene [3].

The present research has utilized PCR-SSCP/HA method. This is one of the best and cheapest methods used to screen large populations. The results primarily confirm the high quality and success of the group in optimizing SSCP conditions. The control group in this research included healthy people with no records of nervous disorders.

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CONCLUSION

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Any defects in sub-units of GABA A receptors pave the way for different nervous problems such as epilepsy. Thus, this receptor is a good starting point for researches and it can be considered a good target point to prepare more effective medicines to treat this disease. This is a preliminary research conducted on the prevalence of epilepsy which has genetic roots associated with GABRG2 gene. The results indicate the relative importance of this gene in the society of epileptic patients of Iran. Although the present research is by no means complete in terms of the pieces studied, this gene can be a good candidate in causing epilepsy and for screening based on the previous study of the research team.

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