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Partial Cloning and Nucleotide Sequencing of Glutamate Decarboxylase Gene Isoform 65 from Human Brain

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Abstract

Background: Gamma -aminobutyric acid (GABA), a non-protein amino acid acts as an inhibitory neurotransmitter in the central nervous system of mammalians. The glutamate decarboxylase (GAD) is responsible for the conversion of L-glutamate to GABA. The human brain has two isoforms of this enzyme, GAD65 and GAD67 that differ in molecular weight, amino acid sequence, antigenicity, cellular location and interaction by factor of pyridoxal phosphate. The purpose of this study was cloning of gene encoding the human glutamate decarboxylase.

Materials and Methods: Total cellular RNA was extracted from human brain tissue and then converted to cDNA. PCR was performed using exclusive primers for gad gene amplification. After purification of PCR product, it was partially cloned successfully in pJET1.2 blunt t-vector and was sent for sequencing.

Results: The outcomes indicate that only gad gene was cloned partially. The length of human gad gene isoform 65 is 1759 base pair that encodes 585 amino acids. The length of partially cloned gad gene in this study was 385 base pair.

Conclusion: Because obtaining fresh human brain is difficult and amount of mRNA is low, it may not be easy to clone full length of human gad gene. The approach described in this paper may be useful in cloning of other genes for which the corresponding mRNA is present at low levels.

Keywords: Glutamate decarboxylase; Gamma -aminobutyric acid; Cloning

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Introduction

Gamma aminobutyric acid (GABA) is a non-protein amino acid that could be found in majority of organisms including plants and animals. GABA with C4H9NO2 formula acts as an inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates and invertebrates (1, 2). GABA as an inhibitory neurotransmitter is essential for normal function of brain, neuronal activity, forming of neuronal network events, and coordination of this network (2). GABA also has roles in development and differentiation of mammalian cells. In addition to CNS, GABA exists in tissues such as pancreas, pituitary gland, testis, gut, ovaries, placenta, uterus, and adrenal gland (3). The glutamate decarboxylase (GAD) is responsible for the conversion of L-glutamate to GABA. GAD catalyzes an α-decarboxylation reaction of l-glutamate to produce GABA (4).

The brain has two isoforms of this enzyme; GAD65 and GAD67 that differ in the molecular weight, amino acid sequence, antigenicity, cellular location and interaction by factor of pyridoxal phosphate (5, 6). GAD65 and GAD67 are derived from the two genes. GAD67 protein is a soluble polypeptide and hydrophilic that is found in cell body of neurons and cytosols of other cells such as pancreatic bête cells. GAD65 is hydrophobic and in comparison to GAD67 is less soluble (5). Posttranslational modification of GAD includes addition of palmitoyl, phosphate groups and protolithic cutting off. Palmitoyl modification of GAD regulates transferring of GAD65 into synapsis (7).

Full length GAD65 has been cloned from human brain cDNA (6) and islets (8). Since GAD convert glutamate to GABA, it could be used for industrial synthesis of GABA using bioreactors such as bacteria and eukaryotic cells. Expression of GAD65 could

also be different in normal and abnormal human brain. Our aim therefore, was cloning of gene encoding the human glutamate decarboxylase from brain tissue to determine whether a full length GAD65 could be cloned from human brain tissue.

Materials and Methods

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

In this study, human brain tissues (pathological leftover samples) were collected from al-Zahra hospital under approved guidelines set by Isfahan University of Medical Sciences. Total cellular RNA was isolated from brain tissue (70-80 mg), using RNXTM plus solution (CinnaGen, Tehran, Iran) and RNeasy Mini Kit. The purity and integrity of the isolated total RNA were evaluated by optical density measurements at 260/280 nm ratios and TAE agarose gel electrophoresis by visual analysis of the 28S and 18S rRNA bands. RNA samples with purity in range of 1.7 to 2 (260/280 nm ratio) were used for reverse transcription. First-strand complementary DNA (cDNA) synthesis reactions were performed using 1 μg DNase (Fermentas)-treated total RNA from each sample and cDNA synthesis kit (Fermentas) with oligomer (Fermentas) priming in a 20 μl reaction according to the manufacturer's instructions. Amplification products were stored at -20 ^{0}C.

Table 1. Primers designed for human gad gene cloning.

Primer	(5'-3')	Restriction enzyme	Tm (^{0}C)	Product Length (bp)
Gad 65 F1	TAAGGATCCCCAAAGCCGATGGCATCTC	*Bam*HI	70.2	1811
Gad 65 R1	GCTGGGAATTCGGAACAGCTTGGTGAGCA	*Eco*RI	72.8	
Gad 67 F1	TAAGGATCCTGATGGCGTCTTCGACCC	*Bam*HI	68.2	1802
Gad 67 R1	GCTGGGAATTCAAACTCATGTTCTGCGAAGGA	*Eco*RI	71.5	
Gad65 F2	CCAAAGCCGATGGCATCTC	-	57.5	1790
Gad 65 R2	GGAACAGCTTGGTGAGCA	-	51.3	
Gad 67 F2	TGATGGCGTCTTCGACCC		56.3	1782
Gad 67 R2	AAACTCATGTTCTGCGAAGGA		53.7	
Gadint F1	TGGCGCCATATCTAACATGTATG	-	72.4	1140 with gad65 R2
Gadint R1	GGCATACATGTTAGATATGGC	-	72.4	720 with gad65 F2
Gad65 int-R3	CACCACATACTGAAGTAAAATG	-	65.7	395 with gad 65 F1
Gad65-intF3	GGTGAAAAGTTTCGATAGATC	-	65.7	385 with Gadint R1
Gad65-intR4	GGAGGCATGCATTTGGTTGCAATTC	-	65.8	546 with Gad65-F2
Gad65-intF4	GAATTGCAACCAAATGCATGCCTCC	-	65.8	535 with gad 65 R1

GAD gene cloning and sequence analysis

To clone entire ORF region of gad gene, primers were designed by oligo7 software. The nucleotide sequences of these primers are illustrated in table 1. PCR reactions were performed in 25 µL vol with 5 µL of cDNA synthesis, PCR Buffer (10X): 2.5 µl, $MgCl_2$ (50 mM): 1µl, dNTP Mix (10mM): 1 µl, Forward Primer (10mM): 1 µl, Reverse Primer (10mM): 1 µl, Taq DNA Polymerase: 0.5 µl, DDW: 13 µl. PCR started with a 3 min denaturation at 94 °C, followed by 30 cycles of 1 min at 94 °C, 30 s -1 min at 58 °C, and 2 min at 72 °C. PCR product was purified using Bioneer Purification Kit (Bioneer, Seoul, Korea). It was then ligated into pJET1.2 T vector system by T4 DNA ligase. In this process, 5 µl 10X buffer with 1 µl of pJET1.2-T vector, 1 µl T4 DNA ligase and 3 µl of purified PCR products (gad gene) were added to a 0.5 ml micro-tube. Next, the micro-tube was incubated at room temperature for 1 hour and then at 4 °C for a day. Only colonies containing external pieces (gad gene) could grow in culture.

Preparation of competent cells from E. coli TOP10 was performed according to the calcium chloride method mentioned in Sambrook book (9) and then the transformation of vector containing the gad gene cDNA to competent cells was done using heat shock as follows.

In a sterile micro-tube, 20 µl of ligation reaction product was mixed with 100 µl competent cells and

was put on ice for 30 min. Then the micro-tube was placed in a hot water bath at 42 °C for 2 min and after heat shock, the sample was placed in a mixture of water and ice (4 °C) for 5 min. One ml of liquid LB medium was added to each micro-tube and it was incubated at 37 °C for 1 hour. After initial incubation and centrifuge in 5000g, the recovered cells were plated onto LB agar plates containing ampicillin, and incubated at 37 °C for 18 hour for screening of colonies. Then some of the colonies were randomly selected as containing the recombinant vector colonies and were inoculated in liquid LB medium containing ampicillin for 24 hours at 37 °C. Then, the recombinant plasmids (pJET1.2 -GAD) were extracted by a plasmid extraction kit (Fermentas) according to its manual.

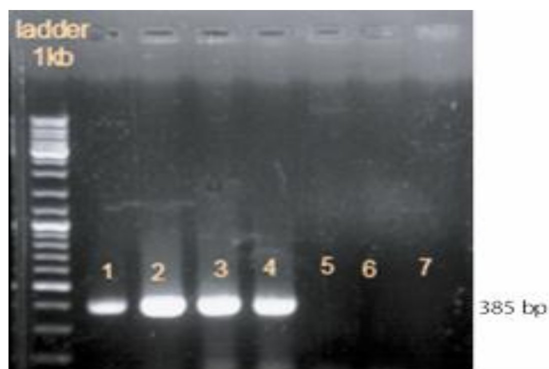

Figure 1. PCR amplification and gel electrophoresis of gad gene in pJET1.2 -GAD plasmid. Lane 1: 1Kbp DNA ladder. Lane 2 to 4: Colony PCR amplification of gad gene in pJET1.2 vector. Lane 5-6: Negative colony PCR. Lane 7: negative control (performing PCR without adding pJET1.2 -GAD plasmid.

Colony PCR and sequencing

To verify the presence of gad gene in recombinant plasmid, plasmid DNA extracted from colonies bacteria was used as a template to amplify the gad gene by PCR performed in 25 µl of solution under the condition previously described. The PCR products were analyzed by electrophoresis. The final validated positive clone of pJET1.2 –GAD was sent to Faza Biotech Company (Tehran, Iran) using T7 forward and SP6 reverse primers for sequence determination.

Ethics Statement

The protocol of this study was approved by Ethic Committee of Isfahan University of Medical Sciences. Every subject signed an informed written consent form.

Results

In order to clone the human gad gene from brain tissue, the PCR method was used as mentioned in materials and methods. The PCR product for the entire open reading frame (ORF) of the gad gene was not obtained by the PCR; instead only partial gad gene 65 was obtained (Figure 1). Grown colonies contain recombinant vector. The gene does not enter plasmid; therefore, colonies would not grow. For more accuracy of gene piece cloning in T/A vector, plasmid extracted from colonies (pJET1.2 -GAD) was used as a template to amplify the gad gene by PCR performed in 25 µl of solution under the condition described previously. Sequencing was done in Faza Biotech Company. The nucleotide sequence of partial human GAD gene consisted of approximately 385 bp. BLAST results showed that partial gad gene from this experiment has 100% similarity with human gad 65 genes (Figure. 2).

Discussion

GABA has different physiological effects such as regulation of blood pressure, tranquilizing, treatment of epilepsy, and inhibition of cancer (10). There are great studies trying to produce GABA by using different glutamic decarboxylase (11). Therefore, cloning of gad genes is very important in both medical and industrial aspects. Differential gene expression of gad is also of great interest, especially regarding human diseases.

Efforts of Yamashita et al. in 1993 only resulted in cloning 600 nucleotide sequence of gad gene using 5 pairs primers (12). Before that, in 1990, Nelson et al. for synthesis of full length GABA transporter gene cloned small pieces of this gene and then using restriction enzymes they fused these gene pieces together (13). Almost all studies about gad gene were done on mouse brain. In mouse it is possible to clone full length gad gene, because the mouse brain could easily be obtained and it is possible to do many experiments on fresh and normal samples. Gad gene cloning was also done in bacteria and other organisms. In current study for cloning of human gad gene several primers have been used (Table 1), but only one pair of primers synthesis a partial 385 sequences of gad gene (Figure 1).

Full length cloning of gad gene is not easy as mentioned above. The reasons for difficulty in cloning of this gene could be unavailable of fresh brain samples, low amount of mRNA, unsuitable primers and so on. In this study we could not able to synthesis the full length of gad gene. Obtaining human brain samples is very difficult especially normal samples. After surgery, the samples are usually put into formalin and extracting RNA from these samples is very difficult since RNA is destroyed immediately. Taq DNA polymerase is really not ideal for cloning, especially not for such a large target. In our case we used Phusion DNA polymerase (NEB) to amplify gad gene. Even using this enzyme our full length cloning was not

successful. Suboptimal c-DNA could be another reason. In this case using Superscript III could not improve cloning. Sometimes primers do not work well. It is current study using several primers only one set worked by a PCR product of 385 bp. Considering studies done so far and partial sequencing of gad gene it is recommended to use pTRL-GAD65, PGEX-3X-hGAD67, pREP10-hGAD67 that are including gad gene. Although we could not clone full length of human gad gene, results of study could be used for future studies such as real time PCR.

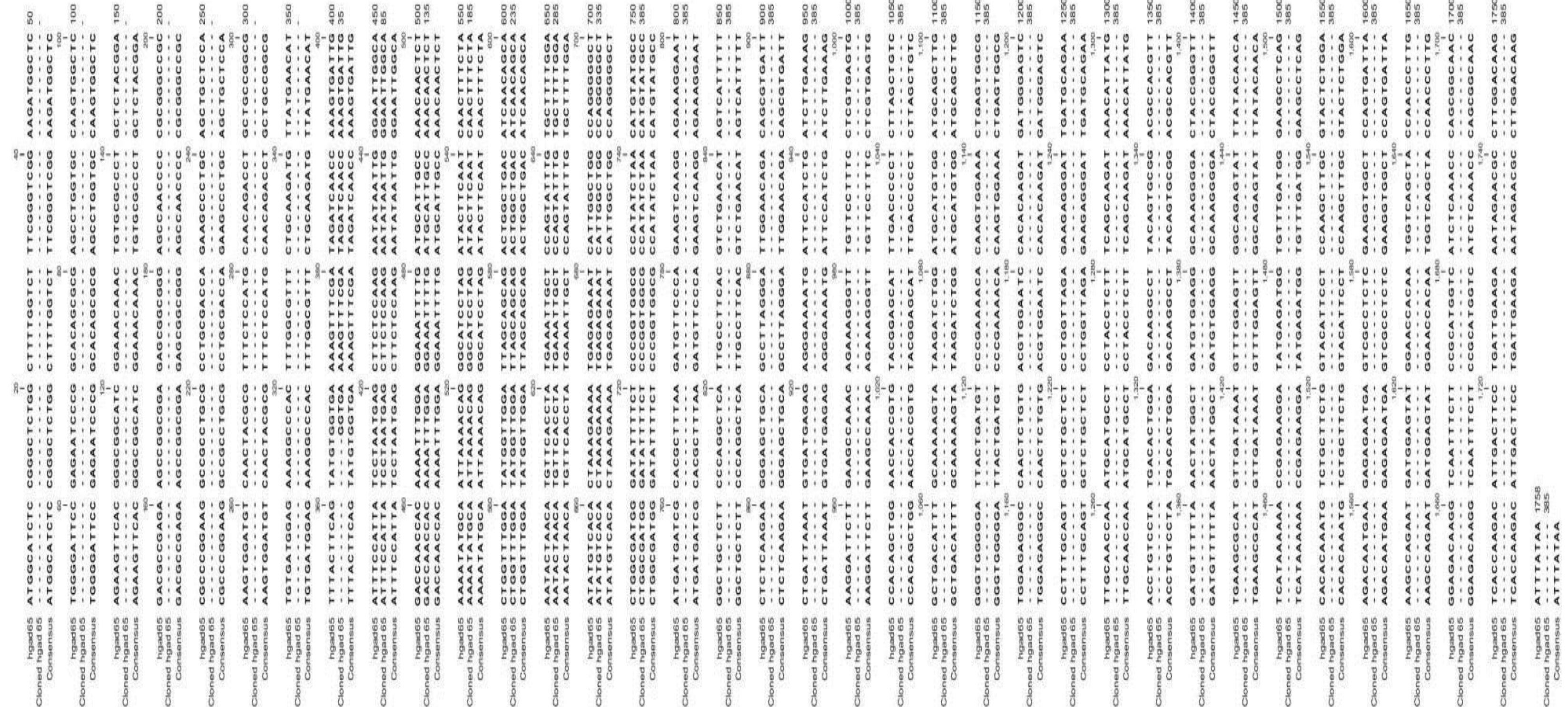

Figure 2. Alignment of the deduced nucleotide acid sequence of human cloned gad gene with its homologues.

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Authors' Contribution
Here are names of people who was responsible for: Esmaeili designed the study, Esmaeili and Dehghani Help in data acquisition, analyzing and inter interpretation. Dehghani wrote the first draft of manuscript

Conflict of interest
No conflict of interest

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