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Mutation analysis of exon 3 and 4 of α-synuclein gene in Parkinson's disease patients from West Libya

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease characterized by losing dopaminergic neurons. The aetiology of Parkinson's disease remains unclear. It is believed that a combination of environmental, age, and genetic factors influence disease pathogenesis. There are few point mutations which have been reported in the α-synuclein gene from PD patients from different ethnic groups (A30P, E46K, H50Q, G51D, A53T, A53E). Here, we collected blood samples from 70 PD patients and 30 healthy controls selected from the west Libyan population that have been matched for ethnic and environmental origin. Genomic DNA was extracted from these samples. Mutation analysis was performed by PCR amplification of the exon 3 and exon 4 of the α-synuclein gene from chromosomal DNA and then sequenced. However, we failed to detect any mutation in exon 3 and 4 including the previously reported mutations. These results suggest that mutation in the α-synuclein gene is an uncommon cause of PD pathogenesis. Furthermore, our data adds further support to the results obtained from worldwide mutation screening in that the α-synuclein gene is a rare cause of PD pathogenesis.

Keywords: Alpha-synuclein; parkinson's disease; neurodegenerative disease.

1. Introduction

There are many neurodegenerative diseases that attack the human nervous system. Parkinson's disease (PD) is the most second common neurodegenerative disease after Alzheimer's disease. PD was described as "Shaking palsy" by Dr. James Parkinson in 1817 [1]. The disease is characterised pathologically by a profound deficiency of dopamine neurons in the basal ganglia and degeneration of substantia nigra, pars compacta, as well as the presence of Lewy bodie(LBs), and Lewy neurites (LNs) [2]. PD is a movement disorder that affects about 0.1–0.2% of the population, but this rate increases to 1.4 % among 55-year-olds and to 3.4 % among 75-year-olds [4]. Most cases of PD are idiopathic without an obvious family history. Although, it is suspected that 70–80% is probably an overestimate for sporadic PD cases and 20–30% is probably an underestimate for familial PD cases [4,5].

The symptoms of PD do not develop until 60 – 70 % of nigral neurons have been lost with an 80% depletion of strial dopamine [6]. A specific PD related problem is the long latency between the first damage to nigral cells and the clinical appearance of symptoms [7]. In the early 1960s replacement therapy (Levodopa)

was developed. Levodopa is still the most effective therapy, although many problems occur with long-term drug treatment. The number of PD patients is increasing and neurodegenerative diseases for example PD, Alzheimer's disease and Motor neuron disease, are projected to surpass cancer as the second most common cause of death among the elderly by the year 2040 [8].

As mentioned before, the aetiology and pathogenesis of PD remains unclear. It has been suggested that PD may be a multifactorial disorder caused by a combination of age, environmental, and genetic factors Figure 1 [9]. A number of candidate genes were investigated to uncover the PD causing mutations in the world population, i.e Mutations in leucine-rich repeat kinase 2 (LRRK2) and alpha-synuclein (SNCA) genes have been associated with PD.

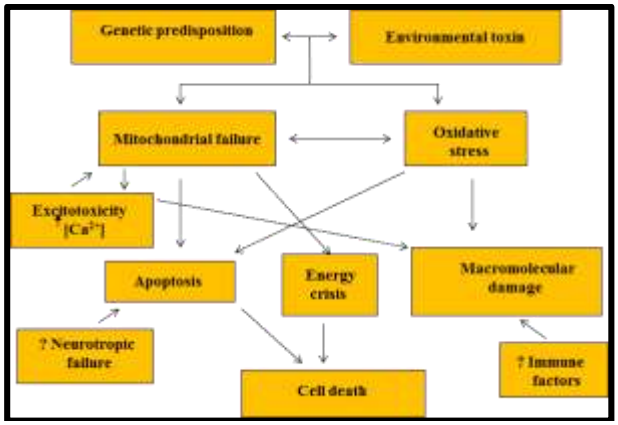

Fig.1. Proposed pathophysiology of dopaminergic cell death in the substantia nigra of patients with PD (Opinion in neurology) [16].

The α-synuclein gene came into the spotlight when it was found to be mutated in different ethnic PD patients (10). Here, mutation analysis for the reported mutations were performed for exon 3 and 4 of the alpha-synuclein gene for PD patients from west Libya population.
Six mutations in the exon 3 and 4 of the alpha-synuclein gene have been reported (A30P, E46K, H50Q, G51D, A53T, A53E) from PD patients from different ethnic groups [1-14,10,15].

The first point mutation was identified by linkage analysis of the Contursi kindred, a large Italian-American family with a highly penetrant autosomal-dominant trait. Sequencing of the α-synuclein gene in affected members of the Contursi kindred revealed a missense mutation in the fourth exon, an A for G substitution at position 209 (G209A). This mutation codes for a substitution of one amino acid, threonine for alanine at position 53 (Ala53Thr) [10].

The mutation Ala53Thr was also found in three small apparently unrelated Greek families (10). A subsequent study found two additional Greek families with PD associated with the Ala53Thr mutation [17].
The second point mutation of the α-synuclein gene was identified in a German family. The mutation results from a single base pair substitution in exon3, C for G at position 88 (G88C), producing a substitution of proline for alanine at amino acid position 30 (Ala30Pro) in the α-synuclein protein sequence [11].

The third point mutation was reported from a Spanish family with autosomal dominant parkinsonism and dementia. Sequencing of the alpha-synuclein gene from that family disorders
showed a a single base pair change at position 188 from G to A (G188A), producing a nonconservative substitution of lysine for Glutamate at amino acid position 46 (E46K) mutation in heterozygosis [12].

The fourth point mutation was reported from a British family with young-onset Parkinson's disease (PD) alpha-synuclein gene. G to A heterozygous mutation at base 152, codon 51 which produces a substitution of aspartic acid for Glycine at amino acid position 51 (G51D) in the α-synuclein sequence [14].
The G51D α-synuclein mutation shows a neuropathological feature of both PD and Multiple system atrophy (MSA).

The fifth point mutation was reported by Proukakis and his colleague [13] (H50Q) in the α-synuclein sequence and proposed that as a pathogenic mutation in the fourth exon, an T to G at position 150 which produce a substitution of glutamine for histidine at amino acid position 50 (H50Q) in the α-synuclein sequence [13].
The sixth point mutation was reported from a Finnish patient with a novel α-synuclein mutation A53E (15). It was as a heterozygous c.158C>A mutation was which detected in the index patient's SNCA gene. This mutation leads to an alanine (Ala) to glutamate amino acid change at codon 53 (A53E).
There have been many other studies searching for further α-synuclein mutations in PD in other ethnic groups, both sporadic and familial PD and in young-onset and old-onset PD patients [18-25], and none of these studies have found more mutations.
An interesting observation about the mutant α-synuclein Ala53Thr in the Contursi kindred is that this mutation is absent in most PD patients, and the normal α-synuclein of rodents and Zebra finches carries threonine at position 53, similar to the mutant α-synuclein of affected members of the Contursi kindred. Nevertheless, the abundance of α-synuclein in LBs and LNs of sporadic PD and Dementia with Lewy bodies

[26,27], with the absence of α-synuclein mutations, suggests that this protein has an important role in the pathogenesis of PD.

Taken together, all of the genetic, pathological and biochemical data support the hypothesis that α-synuclein plays a major role in the pathogenesis of a number of important neurodegenerative diseases, particularly PD.

2. Methods

2.1. Samples

Between 2016 - 2019, Seventy clinically diagnosed PD patients and thirty healthy controls were recruited for this study. Thus, for each case, the diagnosis of idiopathic PD was made by a qualified consultant neurologist, and this diagnosis was made in the presence of two or more of the four cardinal signs of PD: resting tremor, muscular rigidity, bradykinesia, and postural instability (Semchuk *et al*, 1991). Blood samples (EDTA) were collected from PD patients and healthy controls attending an out-patient clinic (Girgarish-Tripoli) at the Neurology Department Spea Hospital.

2.2. DNA extraction

DNA was isolated from each sample using a DNA extraction kit, (Qaigen).

The quality of DNA extracted from all the blood samples used in this study was assessed by agarose (1g) 1% gel electrophoresis.

The concentration of the DNA for all the PD and control samples was also tested by using a Nanodrope machine.

2.3. Optimise the PCR conditions

Before amplification of any region of interest by PCR, it was necessary to optimise the PCR conditions for each reaction. The optimal condition was established by varying $MgCl_2$ concentration, annealing temperature, and the total number of cycles. Mastermixes with $MgCl_2$ concentrations of 1.0mM, 1.5mM, 2.0mM, and 2.5mM were made up. The PCR amplification was repeated with different annealing temperatures of 50°C, 55°C, 60°C, and 65°C and with a different number of total cycles from 25 to 40 cycles.

Electrophoresis on 1.5% agarose gel stained with EtBr was then used to identify the ideal conditions for each reaction by observing the strength and distinct (single band) nature of the amplified PCR product and its correct molecular weight according to that predicted from the previously published sequence data. Subsequently the PCR products derived from exon 3 and exon 4 were subjected to nucleotide sequence analysis using a DNA sequencer machine. The primers used in this study are shown in table 1. Ethical approval was obtained from the Libyan national

committee for biosafety and bioethics (A.B / 151/ 2012).

Table 1. Primers used in this study

Primer name	Conc/ pm/µl	Sequence
α-synuclein exon3$_F$ (Pro1)	50	GTC TCA CAC TTT GGA GGG TTT C
α-synuclein exon3$_R$ (Pro2)	50	AAC TGA CAT TTG GGG TTT ACC
α-synuclein exon4$_F$ (α3)	50	GCT AAT CAG CAA TTT AAG GCTAG
α-synuclein exon4$_R$ (α13)	50	GAT ATG TTC TTA GAT GCT CAG

F-forward, R- Reverse

3. RESULTS

3.1 Collection of samples

One hundred EDTA blood samples (seventy samples were collected from idiopathic PD patients and thirty were collected from healthy controls (Table 2) attending an out-patient clinic (Girgarish-Tripoli) at the Neurology Department Spea Hospital (Dr. Suliman Abod). DNA was isolated from each sample as described in methods.

Table 2. samples

Sample type	Male	Female	Age range
PD	46	24	38 – 83 year
Control	21	9	35 -50 year

3.2 Integrity and purity of isolated DNA

The quality of DNA extracted from all the blood samples was assessed by agarose (1g) 1% gel electrophoresis as described in methods. As shown in figure 2, a distinct DNA band migrating, representing the chromosomal DNA, was observed for all the samples confirming the reliability of our DNA extraction protocol.

Fig. 2. Chromosomal DNA isolated from the blood of PD patients. Lane M contains molecular weight markers (DNA Ladder-PhiX174/Hae III Markers). Lanes 1-19

contain chromosomal DNA samples extracted from PD patients 1-19

The concentration of the DNA for all the PD and control samples was also tested by using Nanodrope machine. The concentration of the DNA was represented 5.1 ng/µl to 140.0 ng/µl for double-stranded DNA (data not shown).

3.1. Screening for mutations in the exon 3 of the α-synuclein gene.

All the samples were screened for the G→C substitution at nucleotide position 88 (G88C) of the coding sequence, which causes an alanine→proline substitution at amino acid position 30 (Ala30Pro) in exon 3 of the α-synuclein gene. Genomic DNA from all PD samples was subjected to polymerase chain reaction (PCR) amplification using the primers Pro1 and Pro2 table 1 [21] which target the exon 3 sequence of the α-synuclein gene as shown in figure 3. The PCR mastermix and conditions were as outlined in tables 3 and 4 respectively.

Table 3. Mastermix Composition for PCR (exon 3) with Pro1 and Pro2 primers

	Per 0.5ml Mastermix (µl)	**Stock Conc.**	**Working Conc.**
dH_2O	423		
NH_4buffer **(Perking Elmer,U.S.A)**	50	10X	1X
$MgCl_2$ **(Perking Elmer,U.S.A)**	15	25mM	1.5mM
dNTP**(Amersham pharmaceuticals)**	5	20mM	200µM
Primer 1**(pro1)**	2	50pm/µl	0.2pm/µl
Primer 2**(pro2)**	2	50pm/µl	0.2pm/µl
Taq polymerase **(Perking Elmer,U.S.A)**	3	5U/µl	3U/100µl

Table 4. Optimised PCR cycling conditions for (exon 3) mastermix

Mastermix	**Cycle conditions**	**No of cycles**
(Pro1 &Pro2)	96°C/1min;55°C/30sec; 72°C/1min	58.1

The above PCR program begin with an initial denaturation period of 96℃/ 5 min and finish with a final extension at 72℃ for 5 min before being held at 15℃ until sample retrieval.

All PCR products were examined by electrophoresis on 1.5% (w/v) agarose gel stained with EtBr to confirm the amplification and the sizes of the PCR products. A representative example of the amplification results is shown in figure 4. As indicated in figure 4 the size of the amplified products corresponded to the size predicted from the previously published sequence data i.e. 395bp**.** Then**,** nucleotide sequence analysis was performed for all the samples (PD and controls), and each PCR product was sequenced in both directions to eliminate any sequence artefacts and improve the accuracy of the sequencing data. A representative example of the automated read out of PD8 sample when sequenced with the forward Pro1 primer is shown in figure 5.

The sequencing analysis of exon 3 for all samples PD and control did not show the Ala30Pro or any other mutation.

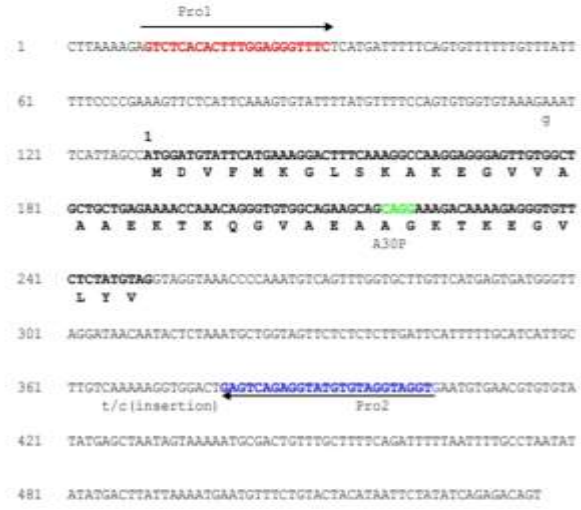

Fig. 3. The α-synuclein gene exon 3 sequence (395bp) from accession number U46897. The forward (Pro1) and reverse (Pro2) primers are highlighted in red and blue respectively, and would amplify the intervening region of 395bp. The direction of the PCR is indicated with horizontal arrows.

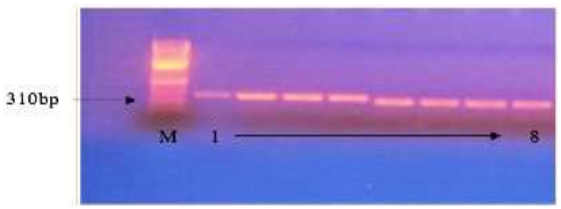

Fig. 4. PCR amplification of exon 3 of the α-synuclein gene (395bp) from PD DNA samples 1-8 using Pro1 & Pro2 primers. Lanes M display molecular weight markers (DNA Ladder-PhiX174/HaeIIIMarkers). Lanes

1-8 display the PCR products amplified from PD DNA samples 1-8.

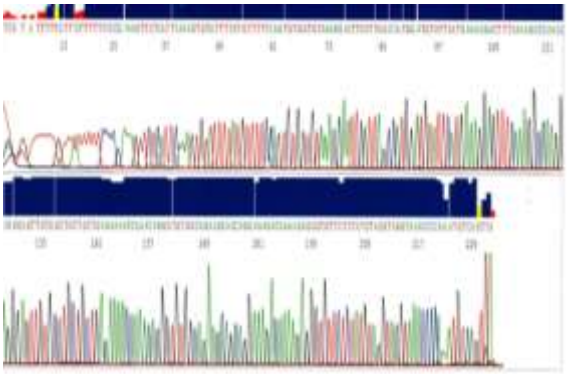

Fig. 5. The nucleotide sequence of exon 3 region of the α-synuclein gene amplified from the DNA of sample PD8 using the forward Pro1 primer.

3.4. Screening for mutations in the exon 4 of the α-synuclein gene.

All the samples were screened for the five reported mutations (E46K, H50Q, G51D, A53T, A53E), in exon 4 of the α-synuclein gene. Genomic DNA extracted from all the PD and control samples was subjected to polymerase chain reaction (PCR) amplification using the primers α3 and α13 Table 1 published by Polymeropoulos [10] which target the exon 4 sequence of the α-synuclein gene. The PCR mastermix and conditions were as outlined in tables 5 and 6 respectively.

Table 5. Mastermix Composition for PCR (exon 4) with α 3 and α 13 primers.

	Per 0.5ml Mastermix (µl)	Stock Conc.	Working Conc.
dH_2O	420		
NH_4buffer **(Perking Elmer,U.S.A)**	50	10X	1X
$MgCl_2$ **(Perking Elmer,U.S.A)**	20	25mM	2.0mM
dNTP**(Amersham pharmaceuticals)**	5	20mM	200µM
Primer 1**(α3)**	1	50pm/µl	0.1pm/µl
Primer 2**(α13)**	1	50pm/µl	0.1pm/µl
Taq polymerase **(Perking Elmer,U.S.A)**	3	5U/µl	3U/100µl

Table 6. Optimise PCR cycling conditions for exon 4 mastermix.

Mastermix	Cycle conditions	No of cycles
(α1 &α13)	96°C/1min;50°C/30sec; 72°C/1min	32

All the above PCR programs begin with an initial denaturation period of 96℃/ 5min and finish with a final extension at 72℃ for 5min before being held at 15℃ until sample retrieval.

A representative example of the amplification results is shown in figure 6. As indicated in figure 6, the size of the amplified products corresponded to the size predicted from the previously published sequence data i.e. 216bp (Figure 7). Nucleotide sequence analysis was performed for all the samples, and each PCR product was sequenced in both directions to eliminate any sequence artefacts and improve the accuracy of the sequencing data. A representative example of the automated read out of PD11 sample when sequenced with the forward α3 primer is shown in figure 8.

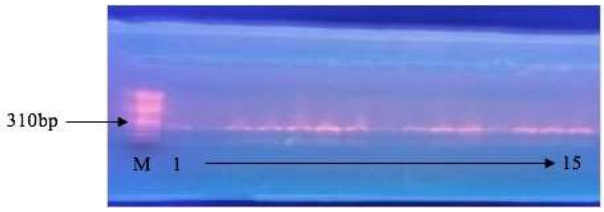

Fig. 6. PCR amplification of exon 4 of the α-synuclein gene from PD. DNA samples 1-15 using α3 & α13 primers. Lanes M display molecular weight markers (DNA Ladder-PhiX174/Hae III Markers). Lanes 1-15 display the PCR products amplified from PD DNA samples 1-15.

Fig. 7. The exon 4 sequence and flanking intron sequences of the α-synuclein gene (10). The forward (α3) and reverse (α13) primers are highlighted in red and blue, respectively and the direction of the polymerase chain reaction is indicated with horizontal arrows. The

flanking intron sequences are shown in italics and the exon 4 sequence is shown in bold font. The translated portion of exon 4 sequence is shown under the sequence in single letter amino acid code that is highlighted dark red.

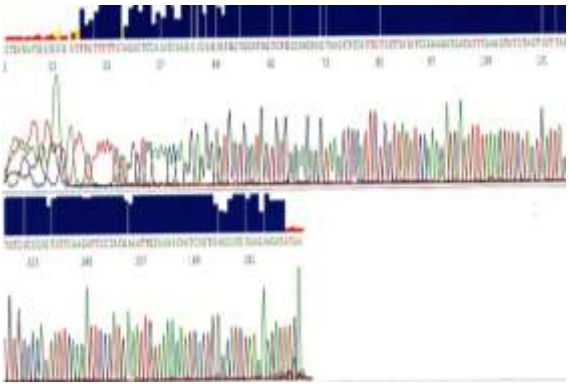

Fig. 8. The nucleotide sequence of exon 4 region of the α-synuclein gene amplified from the DNA of sample PD11 using the forward α3 primer.

4. Discussion

The mutation analysis for exon 4 of α-synuclein gene from all samples (PD & control) were failed to find any mutation.

The pathogenesis of PD remains unclear, and the use of a specific drug or any other therapies proved to be unsatisfactory. The symptoms of PD did not develop until loss of most of nigral neurons, therefore early diagnosis of PD is very important. Furthermore, specific and reliable biomarkers are very important for early diagnosis to predict the disease occurrence and progression. Genetics has been reported as a risk factor associated in the pathogenesis of PD. In fact, more than 20 genes were reported to be involved in the PD etiology (29), so the PD may be present for a long time before the appearance of clinical symptoms. However, PD is expected to be a multifactorial disease and the use of different biomarkers is important at different stages of the disease.

α-synuclein is one of the key genes associated with PD and it is possible to play a central role to its pathogenesis. In this study, mutation screening maddens for the six reported mutations in the exon 3 and 4 of the α-synuclein gene from the West Libyan PD patients.

4.1 Mutation analysis of the α-synuclein gene.

This is the first mutation analysis made for exon 3 and 4 of α-synuclein gene of West Libyan PD patients. We screened all our 70 PD samples and 30 controls for the six-point mutations previously reported in the exon 3 and exon 4 of the alpha-synuclein gene (A30P, E46K, H50Q, G51D, A53T, A53E) from PD patients from different ethnic groups[11-14,10,15].

Sequencing of all the PCR products from exon 3 and 4 of α-synuclein gene from all samples (PD & control) used in this study failed to find any of these mutations in any of the samples examined. Furthermore, we failed to find any new mutation in exon 3 and 4 of α-synuclein gene from all the samples examined. Our results are therefore in agreement with previously published data which have concluded that the mutations of the α-synuclein gene are an uncommon cause of PD[10,24,11,28]. However, our data did not exclude the possibility of presence of other mutations in different regions of α-synuclein gene which could be involved in PD. Furthermore, Libya is known as a heterogenous population (Arab, Barbarian, Tuareg, Tabu, etc,). Therefore, it is necessary to screen a large number of PD samples from all over Libya perhaps within each group to get the significant picture of the relation between the mutations in exon 3 and 4 of the α-synuclein gene and PD in Libya.

Furthermore, α-synuclein protein seems to play a central role in the pathogenesis of PD, and α-synuclein oligomers and aggregates are possible toxic for neurons by inducing neuroinflammation.

Also, formation of intracellular inclusions from α-synuclein or its oligomers lead to a few future neuro-diseases. Many research groups have reported that lesions similar to those found in the human synucleinopathies can be created in transgenic animals expressing high levels of human α-synuclein. These animals progressively develop a loss of dopaminergic cells in the substantia nigra, together with motor abnormalities consistent with synucleinopathies [30,31,32,34]. Furthermore, these studies provide evidence for the central role of α-synuclein deposition in the pathogenesis of these diseases. This suggests that inhibition of α-synuclein aggregation may be a viable strategy for therapeutic intervention in PD and related disorders.

5. Conclusion

The absence of any mutation in this study may from the small number of samples analysed. Therefore, use of large number of PD samples from the whole Libya may show clear picture for the presence of these mutations or not in Libyan population.

Generally, our study shows that the α-synuclein mutations are rear cause of PD, and α-synuclein may induce different effects which influence the functions of neuron cells.

6. Acknowledgements

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