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Original Article

Mutation analysis of RING1 domain of Parkin in early onset of Parkinson's disease in Pakistani patients-a pilot study

Sadaf Niaz[1], Masroor Ellahi Babar[2], Tanveer Hussain[2], Asif Nadeem[1], Misbah Hussain[1], Riffat Mehboob[3*], Fridoon Jawad Ahmad[3]

[1]University of Veterinary and Animal Sciences, Outfall Road, Lahore-54000, Pakistan.
[2]Department of Molecular Biology,Virtual University of Pakistan, Lahore.
[3]Department of Biomedical Sciences, King Edward Medical University, Lahore, Pakistan

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Abstract
Parkinson's disease (PD) is considered as second most common neurodegenerative disorder, occurring mostly in men. Almost 9 genes have been reported to be involved in the progression of PD, if mutated. Among them, PARK2 gene is involved in 50% of the early onset cases. Aim of study was to do mutation analysis of RING domain of Parkin gene as there is no mutation reported previously. In current study, sequence analysis of RING1 domain of Parkin protein was performed in a sample set of 30 patients (selected from different areas of Punjab, Pakistan) to find out any Single Nucleotide Polymorphism (SNP).No SNP was detected in RING1 domain that could be related to the disease. The data suggests that no genetic predisposition in RING1 domain may be responsible for the occurrence of disease in local population. It may be due to genetic changes in any other part of the gene or due to other environmental factors. However, we have limitation of sample size due to less occurrence of disease in this area. The current study is very important for successive researches in Pakistan as the disease ratio is increasing continuously due to consanguineous marriages.
Keywords*:* PARK2gene, RING domain, Parkin gene, Parkinson's disease, early onset of Parkinson's disease

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INTRODUCTION

Parkinson's Disease (PD), a neurodegenerative disorder, contributes approximately 2% of the world's elderly population each year(Solayman *et al.,* 2016). Men are at greater risk for Parkinson's disease than women for a reason unknown (Savica *et al.,* 2016). Loss of dopaminergic neurons can cause this disease (Kahle and Haass, 2004), affecting approximately 6.3 million population worldwide (EPDA, 2016). Although average age of onset of PD is above 60 years but 5-10% cases arise before 50 years and are considered to be early onset of Parkinson's disease (EOPD) and it's incidence has been increasing (Ylikotila *et al.,* 2015). PD is multifactorial involving environmental and genetic factors. Symptoms begins to appear after 50-60% neurons are damaged (Gao and Hong, 2011).

The siblings of PD patients have risk ratio of 1.7 to develop the disease. Several monogenic gene forms have been found to cause PD (Fung *et al.,* 2006). Clinical features of PD were described in 1817 (Fahn, 2015). Although, motor symptoms of the disease begins from one side but later spread to other side of body as well (Perlmutter, 2009). Non-motor symptoms of the disease include abnormal sleep patterns, stress, olfactory deficits and psychosis (Simunovic *et al.,* 2010).

Majority of the people with PD do not exhibit a familial history. Genetic links have been found inEOPD (Ylikotila *et al.,* 2015). Mutations in 9 genes were found to be associated to it. The genes are SNCA (α-synuclein), LRRK2 (leucine-rich repeat kinase 2), PARKN, DJ1, PINK1 (PTEN induced putative kinase 1), UCH-L1 (ubiquitin carboxyl-terminal esterase L1), NR4A2 (nuclear receptor subfamily 4, group A, member 2), MAPT (microtubule-associated protein tau**)** and ATP13A2 (Bischoff, 2011).

The PARK2 gene has been reported on long arm of chromosome 6 (6q25.2-q27),

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*Corresponding author: mehboob.riffat@gmail.com

inherits in autosomal recessive pattern, having 12 exons and encoding 465 amino acid long Parkin protein (Fig. 1). MutationsinPARK2 gene were suggested to be responsible for PD. Most of the mutations reported in PARK2 gene are associated with EOPD (Kitada *et al.,* 1998).

Parkin protein has4 motifs which are conserved: Ubiquitin-Like (UBL) domain, In Between-RING (IBR) motif and 2 RING (Really Interesting New Gene) motifs linked by cysteine rich IBR motifs (Abbas *et al.,* 1999). RING1 (Really Interesting New Gene) domain is encoded by 6th, 7th and a small part of 8th exon (almost 8 bp). Large number of point mutations, deletions and duplications has been reported in this domain and is considered as one of the hot spot regions of the gene. However, function of the domain is not completely explored, it has been found to interact with E2 ubiquitin conjugating enzymes (Nuytemans *et al.,* 2010).

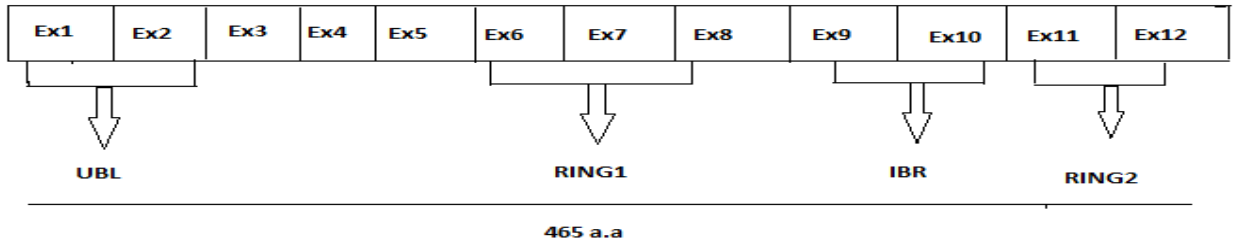

Figure 1: Structure of PARK2 gene and its product Parkin domain

RING1 is a cysteine rich highly variable motif than other domains. Zn ions are assumed to be the modulators of RING1 domain which on binding, enhances the catalytic function of gene and changes the microenvironment of active site. Disruption of zinc binding sites after being mutated may lead to disruption in ubiquitin ligase activity by affecting the stability of Parkin protein (Rankin *et al.,* 2011).

Few PARK2 mutations are associated with aggresomesor lewy bodies formation within cells. High oxidative stress is the main reason of degeneration of neurons by PARK2 mutations(Kahle and Haass, 2004). PARK2 gene mutations are responsible for causing half of the PD. Parkin protein is present in cytosol, golgi complex, synaptic vesicles and endoplasmic reticulum (Mata *et al.,* 2004).

MATERIAL AND METHODS

Patient selection

The patients were selected from different cities of Punjab. Age at onset was main inclusion criteria for patients because PARK2 gene is involved in EOPD (Early Onset of Parkinson's disease). Age at onset was defined as when symptoms of the disease were initially noted. All patients with at least one or more disease symptoms including tremor, Bardykinesia, dementia, postural instability or sleeping disturbances were selected. Most of selected individuals had tremors and muscle rigidity. All patients (N=30; Males=25, Females=5) with age from 21 to 50 years were queried about family history and clinical data.

Table I: Sequence of primers

Exons	Forward primer	Reverse primer	Melting temp (Tm)
Exon 6	GGGAAAGGTTTGATGCTGAT	CTTTGCACAGAGCACAGTCT	58.98°C, 59.45°C
Exon 7	GTTCACTGAGGAAGGCTCGT	TCCTTCATTCCCCAGAACT	56.73°C, 57.05°C

Only 5 patients reported family history for PD. 33% of patients were with age of above 40 years. The study was in accordance with the declaration of WMA (2004). Approval for the study was obtained from the review board of ethical committee of University of Veterinary and Animal Sciences, Lahore, Pakistan.

Experimental procedures

After taking consent from patients, 5ml blood was taken in 5mlvaccutainer tubes (already coated with EDTA) and saved at -4°C (Myhre *et al.,* 2008). DNA was extracted from 500µl blood by following organic extraction method (phenol chloroform isoamyl alchohol

method (PCI)) established by Russel (2006). Quantity and quality of the extracted DNA was checked by nanodrop and agarose gel electrophoresis. Amplification of exons of RING1 domain of PARK2gene was performed by Polymerase Chain Reaction (PCR). PCR amplification of DNA samples was made by sequence specific primers, designed by using PRIMER3 software. DNA sequence of the primers with their melting temperatures is given in the Table I.

PCR amplification of exon 6 and 7 was done for all collected samples. Each PCR reaction was carried out in volume of 25µl that contained 50ng of genomic DNA, 10mMTris/HCl (pH 8.3), 50mMKCl, 1.5mM $MgCl_2$, 200uMdNTPs, 10 pmoles of each primer and 5U Taq Polymerase. PCR conditions included initial denaturation at 95°C for 4 minutes at the start of PCR, then denaturation at 95°C for 30sec following the annealing of primers and extension at 60°C and 72°C respectively. Final extension was done at 72°C for 10 minutes.

Specificity of the PCR product was checked by comparing with 1 kb DNA ladder by agarose gel electrophoresis of the product. After obtaining specific PCR products, they were purified with 80% ethanol at the final concentration to remove any unused dNTP, etc. To check out the variations or point mutations, all the PCR products were sequenced onABI PRISM 3100 Sequencer genetic analyzer by using chain termination method.

RESULTS

In the present study, exons6th and 7th of PARK2 gene encoding RING1 domain of Parkin have been amplified by (PCR) and sequenced by genetic analyzer to find out any point mutation or polymorphism in RING1 domain. By using Bioedit and Chromas software, DNA sequence was read in the form of peaks corresponding to particular nitrogenous bases.

Exon 6

DNA sequence of the affected samples was aligned with the reference sequence to check for the presence of any polymorphism by usingn BLAST software. Reference sequence was obtained from genome browser ENSEMBLE. After alignment of DNA sequences, no polymorphism or variation was found in any DNA sequence. Multiple sequence alignment of the sequencing results was done with the help of ClustalW2 software (Figure 2).

```
1    AGGATAATTAATCCGATTTCTTCTCTTGTCCAAGAGATTGTTTACTGTGGAAACATTTAG 60
2    AGGATAATTAATCCGATTTCTTCTCTTGTCCAAGAGATTGTTTACTGTGGAAACATTTAG 60
3    AGGATAATTAATCCGATTTCTTCTCTTGTCCAAGAGATTGTTTACTGTGGAAACATTTAG 60
4    AGGATAATTAATCCGATTTCTTCTCTTGTCCAAGAGATTGTTTACTGTGGAAACATTTAG 60
5    AGGATAATTAATCCGATTTCTTCTCTTGTCCAAGAGATTGTTTACTGTGGAAACATTTAG 60
     ************************************************************

1    AGGAAAAATGAGCAGCCGGGATCCATGTGTGTGATCATATTTATCTTTCTTTCAGGAATT 120
2    AGGAAAAATGAGCAGCCGGGATCCATGTGTGTGATCATATTTATCTTTCTTTCAGGAATT 120
3    AGGAAAAATGAGCAGCCGGGATCCATGTGTGTGATCATATTTATCTTTCTTTCAGGAATT 120
4    AGGAAAAATGAGCAGCCGGGATCCATGTGTGTGATCATATTTATCTTTCTTTCAGGAATT 120
5    AGGAAAAATGAGCAGCCGGGATCCATGTGTGTGATCATATTTATCTTTCTTTCAGGAATT 120
     ************************************************************
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Figure 2: Clustal 2.1 multiple sequence alignment of Exon 6 by ClustalW2 software. DNA sequence of the affected samples aligned with the reference DNA sequence of exon 6. "*" below the sequence shows the similarity of base pairs.

Exon 7

DNA sequence of amplified 7th exon samples was aligned with the reference sequence by using nBLAST software to locate any variation in DNA sequence of affected individuals.Multiple sequence alignment of sequences was done with the help of ClustalW2 software(Figure 3) which showed 100% similarity between affected and reference sequences.So, no polymorphism or variation was found in the DNA sequence of affected individuals.

PARK2 gene, having 2 million base pairs, is considered as one of the largest genes

in the human genome. Polymorphisms inPARK2gene are the main player in causing EOPD. RING1 is an important domain of Parkin protein. In many cases, there have been found complete deletion of all exons of RING1 domain (Klein *et al.,* 2005). PARK2 gene inherits in autosomal recessive manner that's why both alleles should be altered to show PD symptoms (Medicine, 2013; Trempe *et al.,* 2013).

Figure 3: Clustal 2.1 multiple sequence alignment of exon 7 by ClustalW2software.DNA sequence of the patient's samples aligned with the reference DNA sequence of exon 7. "*" below shows the similarity of base pairs.

It was observed that only 17% of selected patients showed family history for this disease which is similar to the previously reported study (Bekris *et al.,* 2010).Most of the patients were male (n=25). It is reported that men are 1.5 times more at risk to develop PD. The reason behind this increased risk could be more exposure of males to certain hazardous chemicals and mutagens as compared to females in Pakistani population. Secondly, estrogen hormone in females is thought to have protective effect against PD. Oxidative damage is also considered as an important factor in degradation of neurons (Mizuno *et al.,* 2008).

DISCUSSION

PARK2 mutations have been reported in diverse ethnicities with similar relative frequency. Clinical features of PD patients, whether they have PARK2 gene mutations or not, is almost similar. Cognitive differences are less frequently reported. In case of PARK2 mutations, disease progresses slowly (Morrison, 2003). Selected patients also had slow progression of disease, longer disease duration and almost all had motor symptoms of the disease. Hence, according to the results, it may be assumed that no genetic change in RING1 domain is responsible in the progression of PD in this population. In many cases, whole duplication or deletions of the exons have also been observed. A single point mutation in the parkin gene represents a significant risk factor for the occurrence of disease (Periquet*et al.,* 2003). It points towards the fact that there are many factors that lead to PD. The frequency of the RING1 gene mutations in the study is not consistent with the previous studies as no change or mutation has been observed. The novel mutation in UB1 domain of PARK 2 gene already reported in our previous study where one transition C to T mutation has been found in 2nd exon of Ub1 domain which resulted in the amino acid substitution from arginine to cysteine at position 42 (Hussain *et al.,* 2014).

Clinical implication of this study is a step towards the development of diagnostic tests and therapies to control the progression of disease. RING1 domain is very important when PD pathogenesis due to PARK2 mutations is studied. No mutation has been found in RING1 domain in the present study and very few studies are conducted in Pakistan on this issue. Hence, it is very important for future research in Pakistan. The genetic advancements have profound implications for neurologists and patients also. These advances have provided unique opportunities to develop PD biomarkers to improve the diagnostic accuracy for disease management. Specific DNA tests can be developed for diagnostic purpose that would be

helpful in clinical diagnosis at very early stage and further this would lead to the accurate therapy at correct time.

Conclusions

Current study was conducted to find out the variations in RING1 domain by sequencing of exons that could be related to disease. We found no mutation in RING1 domain of PARK2 gene. Such cases are more likely due to complex interactions between environmental and genetic factors and would certainly need a much larger sample size to carry out a sufficiently powered association analysis. It may not be an important causal factor of EOPD in Pakistan but future studies should be done to confirm this.

Competing Interests

Authors declare no conflict of interest

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