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PJMLS

Print ISSN: 2707-4471. Online ISSN: 2707-448X

Research Article	Pak-Euro Journal of Medical and Life Sciences
DOI: 10.31580/pjmls.v4i3.2120	Copyright © All rights are reserved by Corresponding Author
Vol. 4 No. 3, 2021: pp. 195-200	
www.readersinsight.net/pjmls	
Submission: June 15, 2021	**Revised:** July 31, 2021 **Accepted:** September 30, 2021

IDENTIFICATION OF DYSTROPHIN GENE MUTATIONS IN DUCHENNE MUSCULAR DYSTROPHY PATIENTS OF BALOCHISTAN

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INTRODUCTION

Abstract

This study was conducted to identify mutations in the hotspot region of the Dystrophin gene. The patients of Balochistan were selected for the study, diagnosed with Duchene Muscular Dystrophy to check for mutations specific to the local population. A sum of two patients and one control were identified and selected for the study and by their consent Blood was collected. DNA was extracted from these blood samples which were taken in EDTA tubes by using the technique of salt method and PCR was performed to amplify the exon number 3, as it lies in one of the reported regions for having mutations as it is present in the Proximal region of the chromosome. The amplified PCR product was then sent further for sequencing. The results of sequencing concluded that there were no mutations found in this region of dystrophin in selected patients

Keywords: *Duchene muscular dystrophy, Exon 3, Genetic disorder, Mutations*

Muscular dystrophy (MD) is a combination of disorders in which the muscles progressively weaken (1). MD causes Fibroses and fatty replacement in muscles at the later stage of the disease. This disorder is mostly caused by the mutations in the chromosome responsible for coding of Dystrophin (protein) in the muscle cells (1). These mutations in Dystrophin gene cause nine different types of dystrophies, among which Duchenne Muscular Dystrophies (DMD) and Backer Muscular Dystrophies (BMD) are most common which affect the striated skeleton and cardiac muscles (2).

The individual looks like a normal child at birth but having higher levels of Serum Creatine Kinase (Muscle isoform) as compared to normal, the other major symptoms of the disease start to appear from the age of 2 to 5 years. According to the researchers DMD is a muscle degenerating and advancing disease in which individuals usually are unable to walk and therefore become limited to the wheelchair at about 10 years old, and the patient usually dies due to respiratory failure at the age of twenty (3).

On observing the muscles of the individual under muscular biopsies, necrosis and muscular degeneration can be seen even before the clinical symptoms can be observed (4). Although the body tries to repair the damaged muscle fibers, but still the newly regenerated fibers can be seen having a small diameter cytoplasm and large centrally placed nuclei (5) As the muscles continuously degrade the regenerating

power of muscle is eventually lost, resulting in the formation of adipose and fibrous connective tissues instead of the muscle fibers, which results in muscle weakness leading to muscle wasting (6). DMD is caused by mutation in a gene present on the X-chromosome, this gene was identified and named as dystrophin gene, by researchers in 1987 (7). Muscle cells become fragile and break easily in the absence of this Dystrophin protein (8). The Dystrophin gene belongs to a transmembrane protein known as Dystrophin Glycoprotein Complex (DGC) that includes Dystroglycans, Sarcoglycans, Dystrobrevins (9). This gene is expressed in the normal skeleton muscles and is responsible in linking the intracellular actin cytoskeleton and extracellular matrix of the muscles (9).

The gene coding this Dystrophin is located at region X-p21 and is known for its enormous size having 79 Exons with an mRNA transcript size of 14-KB (10). Almost 60% mutations are caused by the deletion of one or more exons along with other types of mutations (11). Thus, so far, there is no available cure for DMD, but some therapies are available that can reduce its effects (11, 12). The main objective of the current research was to study the genetic mutations causing DMD in different regions of the world and compare them to the local population and to find any similarities or differences among them

METHODOLOGY

This study was conducted in Quetta. DMD patients were identified through medical history for confirmation of the disease. Blood samples of two patients and two controls were collected with their consent and all the ethical rules were followed according to the ethical rules of University of Balochistan. The sampling was carried out from patients, accessible in different districts of Balochistan.

Salt method was used for genomic DNA extraction from blood which is a two-day process (13). On the first day RBCs were separated from WBC's (as the RBC's don't contain nucleus / DNA) by mixing the blood with T. E buffer and centrifugation at 4200 RPM for 8 minutes (This step is repeated three times). The WBCs are then incubated with Proteinase K, SDS and TNE buffer over-night at 45 degrees Celsius. On the second day, protein was separated through precipitation with the help of NaCl solution, the DNA was then visualized by the help of chilled Isopropanol and washed using 70% Ethanol to remove any impurities and salt that might have remained in the tubes. This DNA was then estimated using 2% Gel Electrophoresis (13).

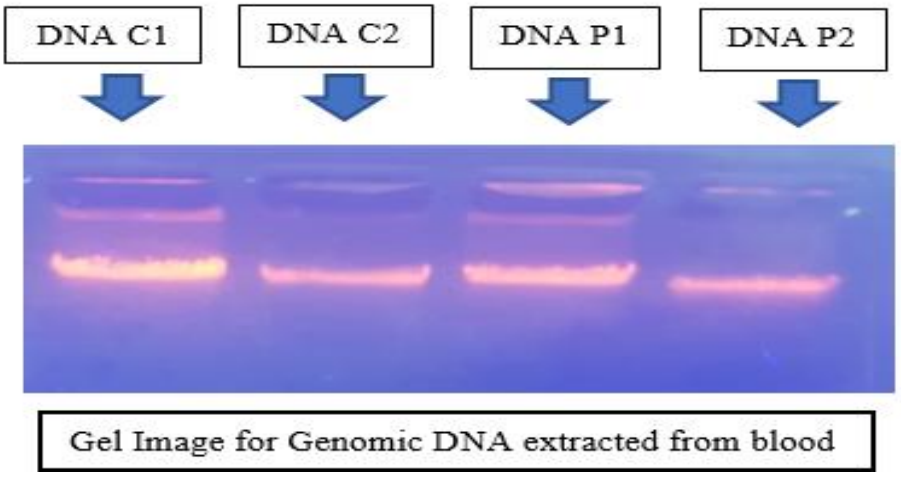

Fig. 1. Gel image for Genomic DNA: Gel electrophoresis is a process to check quality and quantity of DNA which is carried out in 2% agarose gel, Clear bands show the high quality of the DNA while dark bands represent the quantity of it. As the DNA is extracted from blood, it is checked before moving further.

After successful testing of the DNA on Gel Electrophoresis as show in Fig. 1, primers were designed to target the hotspot region prone to mutation in the genomic DNA of patients, for this purpose Exon number 3 was selected which not only lies in the proximal region (14), but is also have been found to have some mutation, selection of this exon was to check if there are any mutations in this region of the gene which might be specific to the region of Balochistan as the target was to find any novel mutations which were not found earlier. One of the main reasons being were very little to no prior research was done in this region. The primer was designed inhouse using the automatic method for which initially the genomic sequence of the Human dystrophin gene was downloaded from National Center for Biotechnology Information NCBI. This genomic data was then used for the synthesis of primer using Primer3 website (15).

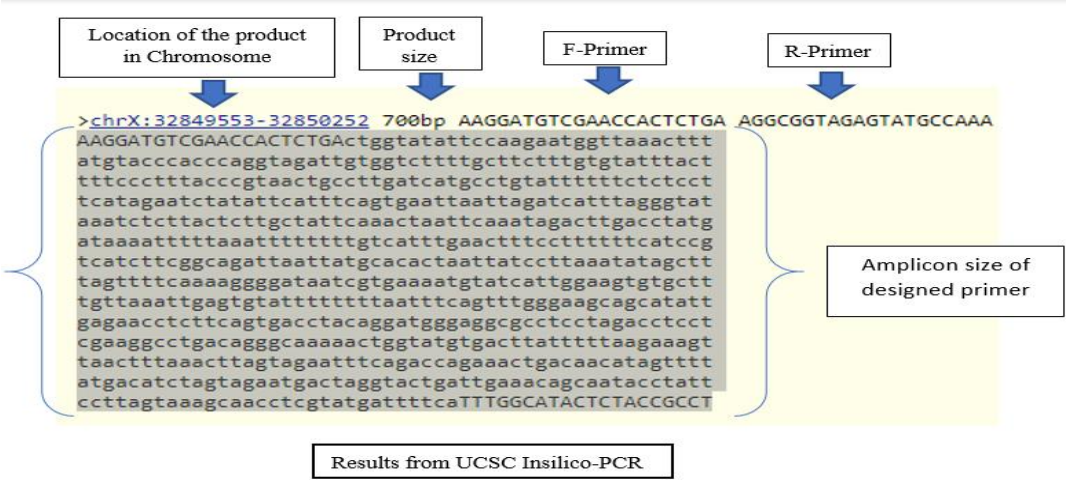

Fig. 2. As the primers were designed in house from the selected region, the primers were then cross verified using the website UCSC Insilico PCR, which not only calculates the annealing temperature of the primer but also shows the amplification product of it. Over-all it reduces the time to do trial and error.

As the method was completely automatic the primers were cross verified using UCSC-Insilico-PCR (16), which not only provides the region which will be amplified using these primers as shown in Figure II, which can be further check using NCBI Blast to check if the Amplified product will be from which region, these primers were then sent to be manufactured by Macrogen located in south Korea. The sequences for primer were as follows.

Forward primer: 5′ -AAGGATGTCGAACCACTCTGA- 3′

Reverse primer: 5′-AGGCGGTAGAGTATGCCAAA-3′

PCR was performed using the above primers of exon number three and the genomic DNA of two patients and one control, exon number three lies in the region prone to mutations in patients suffering from DMD (14). Concentration and quantity of the PCR reagents were used according to the article by Lorenz (17). After preparing the reaction mixture annealing temperature for the amplification process was set (5 degrees below) according to the GC content of the primers which was found out by the automatic method using UCSC In-silico PCR as it not only helps in calculating the annealing temperature as well as calculate and predict the product size as well (16). The PCR product was assessed using 2% agarose gel on electrophoresis to cross verify if the amplified product is of desired size and is the actual region of exon three or not. For which 100 base pair DNA leaders was used along with the PCR amplified product, the results came out to be accurate as the amplified product was of precise 700 base pair. The PCR product was then sent for sequencing.

RESULTS

The PCR product was run on 2% agarose gel using Electrophoresis. 100 base pair ladder was used to check the amplified PCR product size.

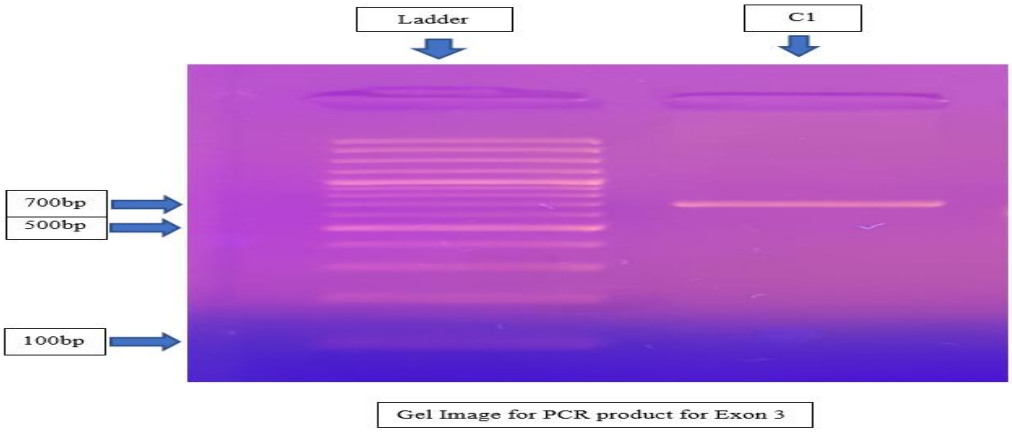

Fig. 3. To verify the accuracy of the amplified product it is checked on gel electrophoresis using 2% agarose gel, the product size is found by comparing it to a ladder which is of known size in this case 100kb ladder was used as a single band represents 100kb increasing in upward direction, as the amplified product is equal to the 7th band, it represents the amplified product is of 700 base pair.

The band size of PCR product was exactly 700 bp which shows that there are no major delectation or duplications in this region of exons number three in our patients otherwise there would have been some difference in the product size as show in Figure. III. There was total 3 samples 1 control and 2 patients which were successfully amplified by PCR, this PCR product was further sent to Beijing Genomic Institute (BGI Hong Kong), for sequencing to check for any SNP's but there were no mutations found in this region of exon number 3 (Figs. 4, 5, 6).

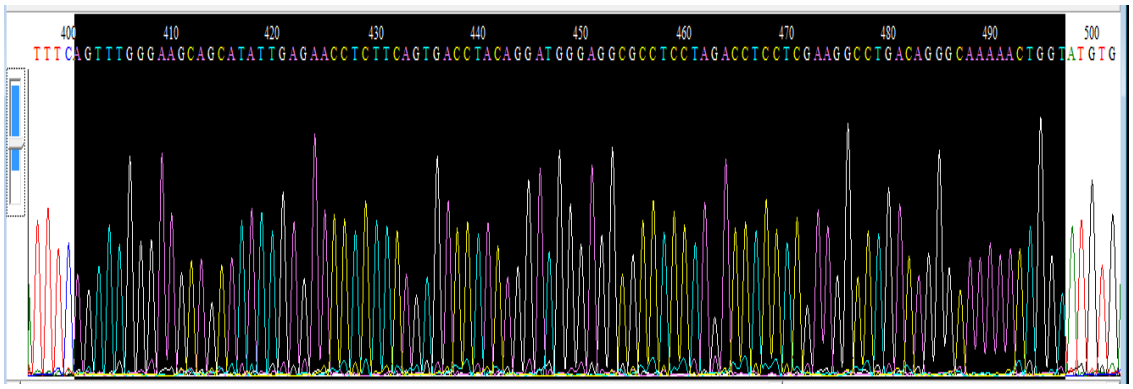

Fig. 4. The above image shows the sequencing results for the Patient 1, as the bands are clear with clear peaks and no overlapping it represents there were no mutations such as deletion duplication etc.

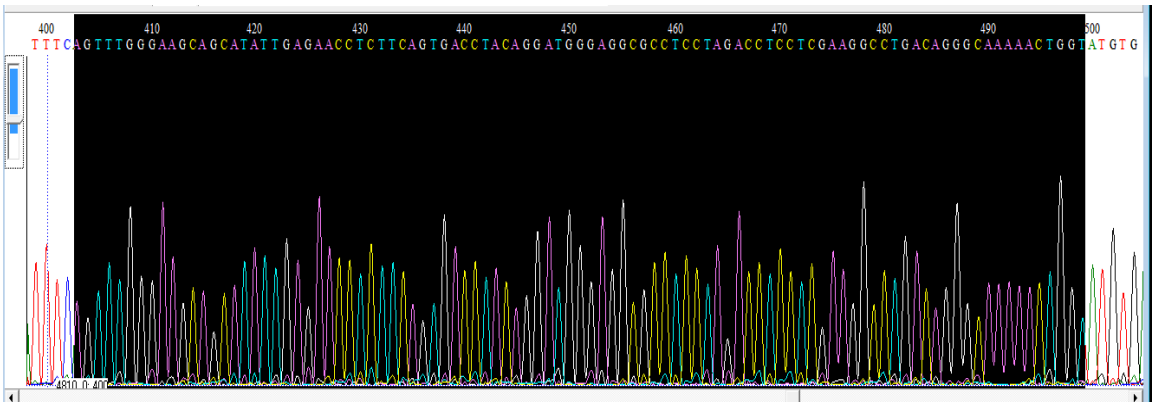

Fig. 5. The above image shows the sequencing results for the Control 1, as the bands are clear with clear peaks and no overlapping it represents there were no mutations such as deletion duplication in control as well

Fig. 6. The above image shows the sequencing results for the Patients 2, as the bands are clear with clear peaks and no overlapping it represents there were no mutations such as deletion duplication in patients 2 as well.

The results were analyzed using the Bio-edit which is free software found online. It was used to check the quality of bands and if there were any overlaps or double bands which gives a clue for any mutation of errors in results. The bands were clear and of high quality and there was no overlapping found as shown in figure 4, 5 and 6. These results were further analyzed for mutations or errors using a function NCBI called BLAST, which compared the given query to its database as shown in Figure VII, as a result no mutations or deletions were found.

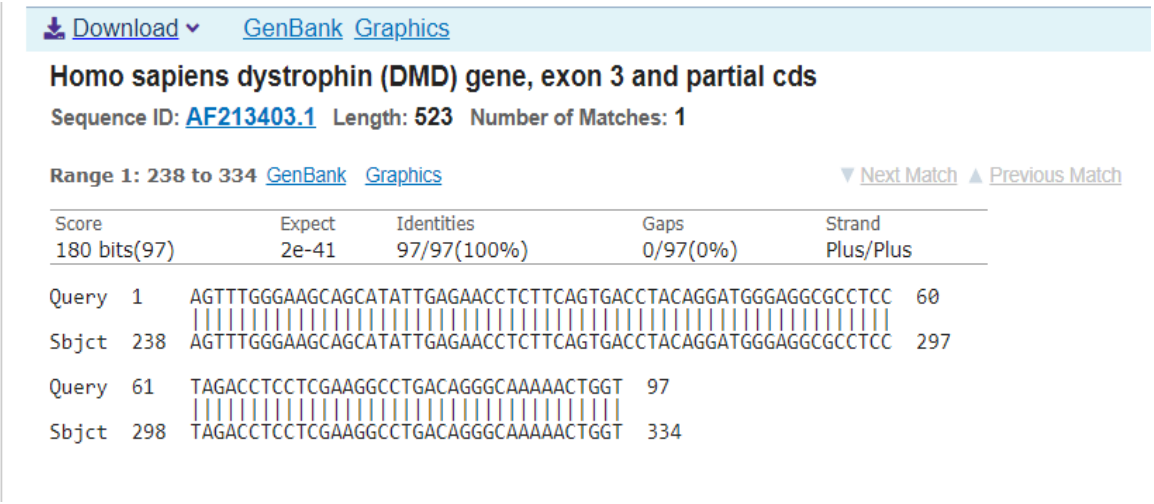

Fig. 7. The sequencing results were then cross verified using NCBI (16) Blast, which not only compares the sequence in its database but also helps in identifying any mutations which could not have been seen prior using bio-edit tool

DISCUSSION

As DMD is a muscular degenerative disease, which is caused by mutation in the dystrophin gene. According to an article published by Chen who conducted research for the screening of mutations in DMD patients of China. A total of 119 patients enrolled for this study and were screened for mutations in DMD gene, Chen found a wide variety of mutations such as SNP's, deletion, and duplications in these patients, out of which most common was deletion, Chen also identified two distinct regions for mutations starting from exon 3 to 21 and exon 44 to 53 (14).

In another article by Srivastava, the mutations in the DMD gene are distributed in two regions one in the central region including exon number 43-51 while the other region is the proximal region including exon number 2-19. According to Srivastava the main cause of the development of disease in an individual was deletion mutation of one or more than one exon of the DMD gene (18). Also, in an article by Neri M publish in 2020 conducted research on Italian population consisting of 1,162 DMD patients also stated that the major region for mutation is clustered to the region of exon 45 to 55 and 2 to 10 (19). Which is accordance with the previous data and in light of this data provided by these articles it was decided to conduct a study which compares the mutations occurring in patients of different regions of the world and try to find any similarities or differences among them?

CONCLUSION AND RECOMMENDATIONS

DMD gene contains 79 exons which are divided into two distinct regions which are distal and proximal region. As mentioned earlier these regions are specific for mutations which range from exon number 3-23 for proximal region while exon number 43-54 lies in the distal region (18-20). After conducting the research there were no novel mutations found in the DMD patients of this region. This might be due to the small sample size or limited study to exons. To find mutations specific to the region of Balochistan it is highly recommend conducting a study where whole gene or at least complete amplification of hotspot regions on this gene to be amplified and sequenced, which will contribute to the knowledge regarding this disease but also will help the community of scientists to better understand the disease and also understand how it works and effects population of different regions of the world. Or perhaps one day this information might help us to eradicate this deadly disease or at least minimize its effects on the individual and to the community.

Acknowledgement:

I would like to thank my teacher Dr. Rozeena Shaikh as she is one of the main contributors, without her help this research would have not been possible, they not only guided me with best of their knowledge as well as provided all the necessary resources required to complete this project but groomed me well for the challenges down the road, I will never forget their sacrifices for me.

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