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GENE MUTATIONS ANALYSIS OF CALPAINOPATHY: A LIMB-GIRDLE MUSCULAR DYSTROPHY

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Thesis submitted in partial fulfillment of the 5 Year Dual Degree Programme B. Tech - M. Tech

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

WAKNAGHAT

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CERTIFICATE

This is to certify that the work titled, "**Gene Mutations Analysis of Calpainopathy: A Limb-Girdle Muscular Dystrophy**" submitted by **Kunal Passi and Lavanya Jain** in partial fulfillment for the award of 5 Year Dual Degree Programme B. Tech - M. Tech in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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27th May 2013

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Kunal Passi and Lavanya Jain

27th May 2013

SUMMARY

Calpain, a heterodimer consisting of a large and a small subunit, is a major intracellular protease, although its function has not been well established. This gene encodes a muscle-specific member of the calpain large subunit family that specifically binds to titin. Mutations in this gene are associated with limb-girdle muscular dystrophies type 2A. Alternate promoters and alternative splicing result in multiple transcript variants encoding different isoforms and some variants are ubiquitously expressed.

The calpain-3 gene is predominantly expressed in skeletal muscle tissue as a 3.5 kb transcript. Additional transcripts, derived from alternative splicing and the use of alternative promoter have also been identified.

There were 440 mutations reported in CAPN3 gene till 2011, with a majority of missense mutations. There are many population variations as well. Some mutations are more common in a particular ethnicity or geographical location whereas others are more common in other places.

The human calpain-3 gene (*Gene Symbol* **CAPN3**, alias p94) localizes to chromosome 15q15.1-15.3 and has 24 exons spanning some 53 kb of DNA. The gene is exceptional by having many rather small exons; 10 exons are only 58-86 bp, while exons 12, 15 and 14 are even smaller, 12, 18 and 37 bp resp. Most introns vary in size between 0.2-2.6 kb. Exceptional are introns 18 and 20, measuring below 100 bp, and intron 1, with a size of 24.3 kb covering about half of the gene. Exon 13 is 209 bp long.

This project is a result of the comparison between exon 13 of the diseased and normal genotype.

Kunal Passi and Lavanya Jain

27th May 2013

Dr. Harish Changotra

27th May 2013

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CHAPTER 1

INTRODUCTION

MUSCULAR DYSTROPHY

Muscular dystrophy is a group of inherited disorders that involve muscle weakness and loss of muscle tissue, which get worse over time.

Muscular dystrophies, or MD, are a group of inherited conditions, which means they are passed down through families. They may occur in childhood or adulthood. There are many different types of muscular dystrophy. They include:

- Becker muscular dystrophy
- Duchenne muscular dystrophy
- Emery-Dreifuss muscular dystrophy
- Facioscapulohumeral muscular dystrophy
- Limb-girdle muscular dystrophy
- Myotonia congenital
- Myotonic dystrophy

LIMB-GIRDLE MUSCULAR DYSTROPHY (LGMD)

Limb-girdle muscular dystrophy (LGMD) is a purely descriptive term, generally reserved for childhood- or adult-onset muscular dystrophies that are distinct from the much more common X-linked dystrophinopathies. LGMDs are typically nonsyndromic, with clinical involvement typically limited to skeletal muscle. Individuals with LGMD generally show weakness and wasting restricted to the limb musculature, proximal greater than distal, and muscle degeneration/regeneration on muscle biopsy. Most individuals with LGMD show relative sparing

of the bulbar muscles, although exceptions occur, depending on the genetic subtype. Onset, progression, and distribution of the weakness and wasting vary considerably among individuals and genetic subtypes.

CALPAINOPATHY (LGMD2A)

Calpainopathy is characterized by symmetric and progressive weakness of proximal (limb-girdle) muscles. The age at onset of muscle weakness ranges from two to 40 years. The phenotype shows intra- and interfamilial variability ranging from mild to severe. Three calpainopathy phenotypes have been identified based on the distribution of muscle weakness and age at onset:

- Pelvifemoral limb-girdle muscular dystrophies (LGMDs) (Leyden-Möbius) phenotype, the most frequently observed calpainopathy phenotype, in which muscle weakness is first evident in the pelvic girdle and later in the shoulder girdle with onset before age 12 years or after age 30 years;
- Scapulohumeral LGMD (Erb) phenotype, usually a milder phenotype with infrequent early onset, in which muscle weakness is first evident in the shoulder girdle and later in the pelvic girdle; and
- HyperCKemia, usually observed in children or young individuals, in which asymptomatic individuals have only high serum creatine kinase (CK) concentrations.

Clinical findings include the tendency to walk on tiptoes, difficulty in running, scapular winging, waddling gait, and slight hyperlordosis. Other findings include symmetric weakness of proximal more than distal muscles in the limbs, trunk, and periscapular area; laxity of the abdominal muscles; Achilles tendon shortening; scoliosis; and joint contractures. Affected individuals typically do not have cardiac involvement or intellectual disability.

GENE

CAPN3, which encodes proteolytic enzyme calpain-3, is the only gene in which mutations are known to cause calpainopathy.

The gene for LGMD-2A was mapped to a 1 cM region of chromosome 15q51.1-15.3.

Approximately 80% of individuals with *CAPN3* mutations show variable levels of calpain-3 protein deficiency. Although *CAPN3* mutations are distributed throughout the length of the gene, most are clustered in a limited number of exons; mutation scanning of these regions detects approximately 90% of causative mutations. Sequence analysis of the entire coding region detects approximately 99% of mutations.

MRNA

The calpain-3 gene is predominantly expressed in skeletal muscle tissue as a 3.5 kb transcript. Additional transcripts, derived from alternative splicing and the use of alternative promoter have also been identified.

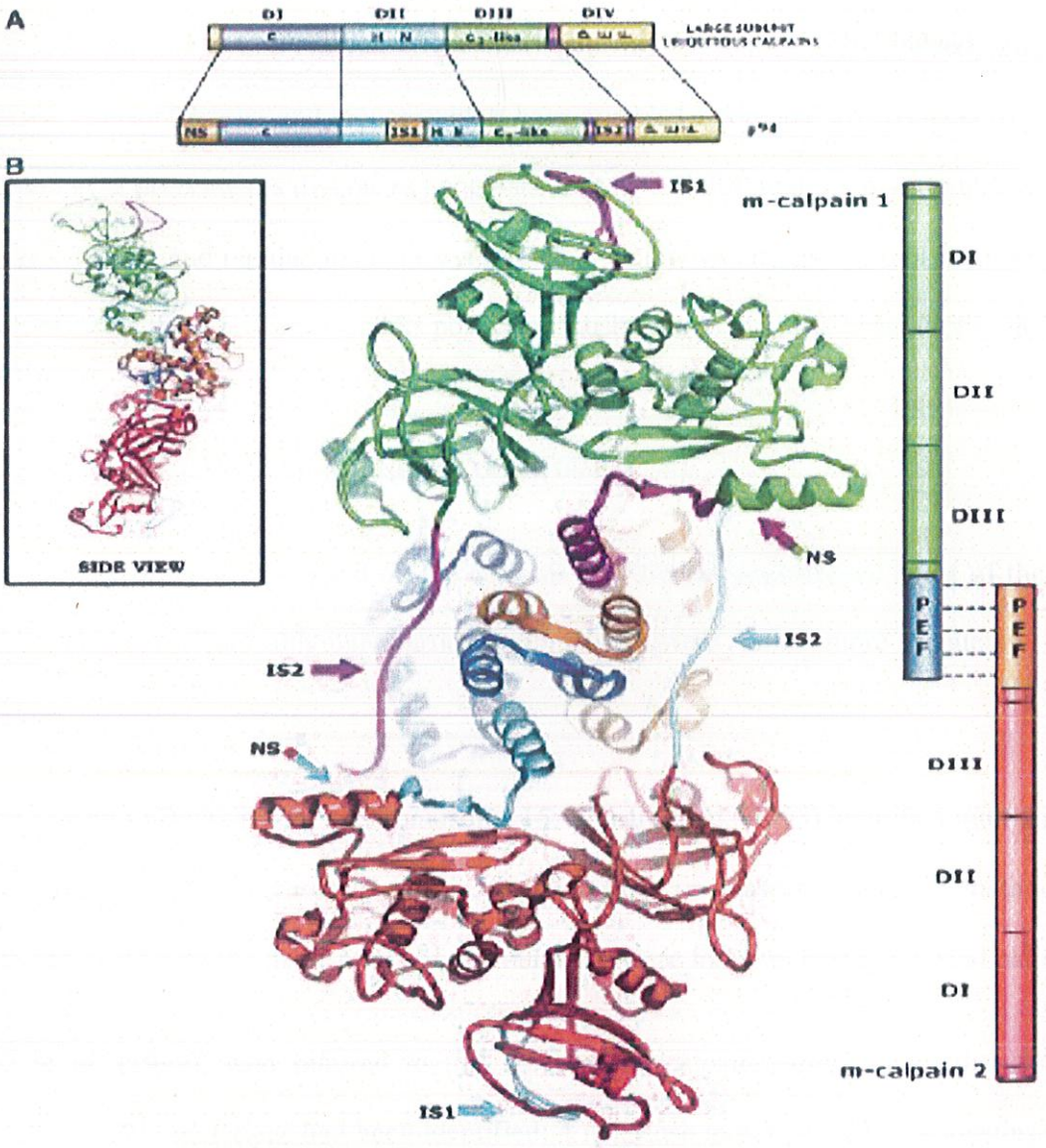
PROTEIN

Calpain-3 (CAPN3) is a muscle-specific member of the calpain family, a group of nonlysosomal calcium-dependent cysteine proteases whose functional role is largely unknown. CAPN3 is a 94 kDa protein containing four main domains and three short unique inserted sequences, NS, IS1 and IS2;

- I - containing NS
- II - a cysteine protease module (with Cys-129 and His-334 constituting the active site) with internally IS1

- III- containing IS2 at the C-terminal end
- IV - a possible Ca^{2+}-binding domain

IS2 contains a titin-binding site and and a nuclear location signal (NLS).



Crystal structure of CAPN3 shown as a homodimer. Shown in panel A is a diagram of the four domains of the large subunit of m-calpain (top) and CAPN3 (bottom). The three additional sequences of NS, IS1 and IS2 are shown in yellow. Shown in panel B is a graphical representation of the crystal structure of two CAPN3 molecules homodimerizing through their C termini. NS, IS1 and IS2 regions are indicated by arrows. To the right of the crystal structure is a bar figure that represents a side view, demonstrating how the two CAPN3 molecules interact through the five EF hand domains. Figure taken from Ravulapalli et al (2005).

FUNCTION OF CALPAIN -3 IN PHYSIOLOGICAL PROCESSES

The exact mechanisms involved in the pathogenesis of LGMD2A are still unknown. Since CAPN3 is a protease with a potentially broad range of substrates, there is a high probability that it is involved in regulating multiple physiological processes. This diverse role for CAPN3 means that its absence or mutations in CAPN3 can affect many pathways in muscle cells. Indeed, studies using cell culture, human biopsies and mouse models have revealed a potential involvement of CAPN3 in the regulation of processes as diverse as apoptosis, muscle cell differentiation, sarcomere formation, muscle remodeling and regulation of the cytoskeleton. Below we discuss current knowledge of the role of CAPN3 in these processes and its possible contribution to the pathogenesis of LGMD2A [23].

CALPAIN-3 SEQUENCE VARIATIONS AND POLYMORPHISMS

There are many observations based on the analysis of different populations. Most of them (70%) represent private variants although particular mutations were found more frequently in some populations.

In families with LGMD2 linked to chromosome 15, Richard et al. (1995) identified mutations in the calpain-3 gene; in all, 15 nonsense, splice site, frameshift, or missense calpain-3 mutations were found to segregate with the disease. Six of the mutations were found in Reunion Island patients.

Richard et al. (1999), who referred to this disorder as 'calpainopathy,' stated that 97 distinct pathogenic CAPN3 mutations had been identified: 4 nonsense mutations, 32 deletions/insertions, 8 splice site mutations, and 53 missense mutations, together with 12 polymorphisms and 5 unclassified variants. The mutations, most of which represented private variants, were distributed along the entire length of the CAPN3 gene.

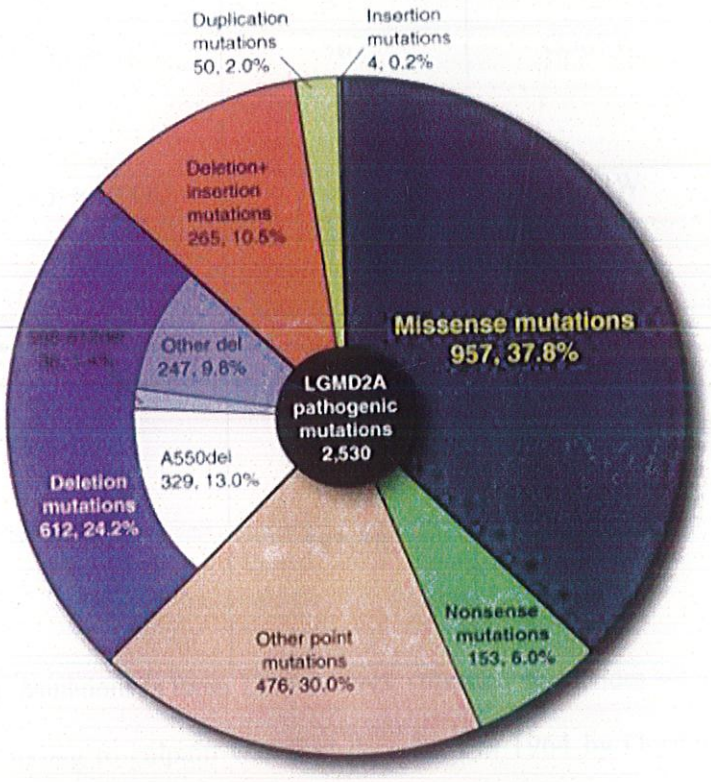
Canki-Klain et al. (2004) found that 550delA was the most common mutation among Croatian patients with LGMD2A, with a prevalence of 76% of mutant CAPN3 alleles. The detection of 4

healthy 550delA heterozygous individuals yielded a frequency of 1 in 133 (0.75%) in the general Croatian population. All 4 carriers originated from an island and mountain region near the Adriatic, indicating a probable founder effect.

Fanin et al. (2005) determined that the prevalence of LGMD2A in northeastern Italy was 9.47 per million. Two founder mutations in the CAPN3 gene, 550delA and R490Q, were identified. Todorova et al. (2007) identified mutations in the CAPN3 gene in 20 (42%) of 48 unrelated Bulgarian patients with muscular dystrophy. Forty percent of the patients were homozygous for the 500delA mutation, and 70% carried it on at least 1 allele.

Limb girdle muscular dystrophies (LGMDs) comprise a clinically and genetically heterogeneous group of diseases usually characterised by progressive muscle weakness and wasting of pelvic and shoulder girdles.[1] Estimates of prevalence for all forms of LGMD range from one in 14,500 to one in 123,000 [van der Kooi et al 1996, Urtasun et al 1998]. These comprise of both autosomal dominant (LGMD1) as well as recessive (LGMD2). Calpainopathy (LGMD2A) is considered the most common form of LGMD [Bushby & Beckmann 2003], representing approximately 30% of LGMD cases, depending on the geographic region [Chou et al 1999, Zatz et al 2000]. Estimates based on molecular data indicate that the frequency ranges from 10% of LGMD cases in a white population from the US [Chou et al 1999, Moore et al 2006], to 13% in Denmark [Duno et al 2008], 21% in the Netherlands [van der Kooi et al 2007], 25-28% in Italy [Guglieri et al 2008, Fanin et al 2009b], 26% in Japan [Kawai et al 1998], 50% in Turkey and India [Dincer et al 1997, Balci et al 2006, Pathak et al 2010], and 80% in the Basque country [Urtasun et al 1998] and Russia [Pogoda et al 2000]. LGMD2A is caused by mutations in the CAPN3 gene, that encodes for a non-structural protein, the enzyme called calpain-3.[2] Calpain-3 is the muscle specific member of a family of Ca^{2+}-dependent proteases, which are supposed to play a part in many intracellular processes, including cell motility, apoptosis, differentiation and cell cycle regulation, by modulating the biological activity of their substrates through limited and strictly controlled proteolysis.[3] While calpain 3

mRNA is expressed at high levels in muscle and appears to have some role in developmental processes, muscles of patients and mice lacking calpain 3 still form apparently normal muscle during prenatal development; thus, a functional calpain 3 protease is not mandatory for muscle to form *in vivo* but it is a pre-requisite for muscle to remain healthy.[5] The human CAPN-3 gene comprises 24 exons and covers a genomic region of 50 kb. It is expressed as a 3.5 kb transcript, and translated as a 94 kD protein.[1] A total of 2,530 mutations, including 456 unique ones, have been reported in the CAPN3 gene so far (2011). One characteristic of these mutations is that more than half (62.7%) of them are nucleotide substitutions, of which missense mutations are dominant (60.3% among all substitutions, and 37.8% among all pathogenic mutations).[4]



Summary of calpainopathy mutation types

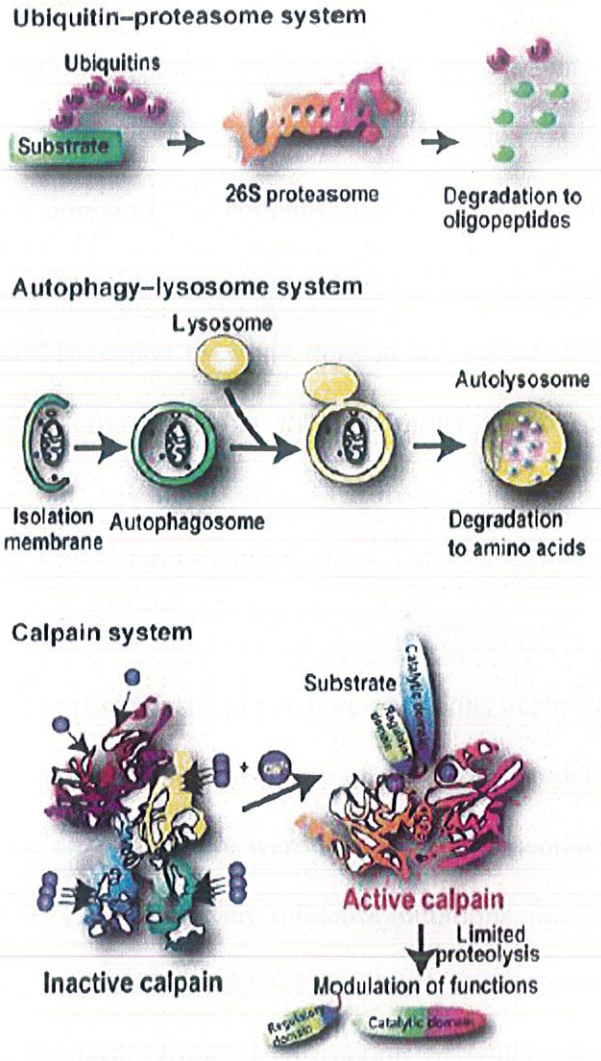
CHAPTER 2

REVIEW

Postion (bp)	Mutation	Position in Amino Acid	Mutation	Reference
1611	TAC → TAA	537	Y537X	Généthon Dinçer et al. (1997), Richard et al. (1997)
1622	CGG → CAG	541	R541Q	Généthon
1699	GGG → TGG	567	G567W	Richard et al. (1997)
1714	CGG → TGG	572	R572W	Richard et al. (1997)
1715	CGG → CAG	572	R572Q	Richard et al. (1995) Généthon
1743/1744	ΔTG	581-582	1743ΔTG	Hospital Natr. Sra. Aranzazu

Table 1. Mutations in Exon 13 of CAPN3. (Mutated nucleotides are underlined)

An enzyme corresponding to calpain was first described in 1964 by Gordon Guroff.[24, 25] Calpains differ from other major intracellular proteolytic components such as proteasomes[45] and lysosomal proteases functioning in autophagy;[46] these systems eliminate and recycle their substrates by degradation. Calpains act by proteolytic processing, as in the activation of conventional protein kinase C (PKC). Calpains are unique in that they directly recognize substrates, whereas proteasomes and autophagy rely on other systems—ubiquitylation and autophagosome formation, respectively—to tag their substrates (Figure).



Major intracellular proteolytic systems. The ubiquitin-proteasome system degrades and eliminates specific substrate proteins with an ubiquitin-tagging system consisting of >1000 ubiquitin ligases. The autophagy-lysosome system primarily degrades non-specific cell components, including proteins and microorganisms, contained by isolation membranes. The caspase system is a major intracellular proteolytic system with primarily apoptotic functions. In contrast, calpains primarily elicit proteolytic processing, rather than degradation, to modulate or modify substrate activity, specificity, longevity, localization, and structure.

Calpain 3 (CAPN3) also belongs to this family of Ca^{2+}- activated neutral cysteine proteinases that have been identified in a wide variety of organisms as disparate as humans and worms.[6, 7] It has 54 and 51% sequence homology to the 80 kDa subunits of μ- and m-calpains, respectively, and shares similar properties with these ubiquitously expressed calpains such as Ca^{2+}- dependent activation and maximal activity at neutral pH.[6] At the same time CAPN3 also has distinct characteristics which

distinguish it from the ubiquitous calpains.[8] First, CAPN3 is predominantly muscle-specific [9], but is detectable in lens, liver, brain and cardiac muscle during development. [10–13] Also, CAPN3 lacks a small subunit and likely functions as a homodimer. [14–18] CAPN3 has some unique domains including its NH2-terminal domain I that contains 20–30 additional amino acids not present in μ- and m-calpains and two unique 'insertion sequences' of 62 and 77 amino acids at the COOH-terminal regions of domain II (called IS1) and domain III (called IS2). In addition, the calcium concentration essential for activation is in the nanomolar range (compared with micro and millimolar concentrations for calpain 1 and 2, correspondingly).[19] Finally, CAPN3 is very unstable[18, 20] and is subject to fast autoproteolytic degradation, a feature which has made it difficult to thoroughly characterize.

In contrast to other types of muscle dystrophy, where mutations occur in genes encoding structural proteins, calpainopathy was the first reported type of dystrophy predetermined by mutations in a gene encoding a proteolytic enzyme. There were over 440 documented mutations in the calpain 3 gene till 2011, among them 212 (~50%) were missense mutations, many of which alter its catalytic activity.[21] CAPN3 is a component of the skeletal muscle triad, responsible for calcium release. It is also a component of the dysferlin complex, disruption of which also results in a limb girdle dystrophy.[22] It is plausible that the subcellular localization of CAPN3 determines its particular cellular function, and that mutations can affect any of those functions directly or indirectly.[6] In addition, the deleterious effect of missense mutations can arise not only from direct disruption of CAPN3 function(s), but also from disruption of the protein's structural integrity, which could affect its intra or intermolecular protein interactions and lead to decreased stability or altered localization. It has been assumed that titin regulates CAPN3 activity and prevents it from autoproteolytic degradation.[8, 23]

Severity of LGMD2A can vary considerably even within a family. The onset of the disease is usually in the second decade of life but was reported to occur as early as 2.5 years and as late as 49 years of age. Predominant symmetrical and simultaneous involvement of pelvic and scapular girdle

and trunk muscles without facial, oculo-motor or cardiac involvements is typical for LGMD2A.[26, 27] However, the course of disease progression as well as muscles affected by the disease can differ between LGMD2A patients. Characteristic histopathological features of LGMD2A include necrosis and regeneration, fiber diameter variability and/or atrophy.[28-30]

In sarcomeres, calpain-3 directly binds to connectin/titin at the M-lines and the N2A regions[8] where multiple proteins form complexes to respond to myofibrillar signal transduction systems.[31, 32] Interestingly, calpain-3 changes its localization from the M-lines to the N2A regions as sarcomeres extend.[33, 34] Mobility of calpain-3 between the M-lines and the cytosol becomes compromised when its protease activity is lost.[33]

Depolarization of the muscle cell membrane caused by the opening of acetylcholine receptors results in the cell excitation-coupled contraction. Intracellular $[Na^+]$ ($[Na^+]_i$) is maintained at rather low levels (~15 mm). To achieve this, cells express Na^+/K^+-ATPases on their cytoplasmic membrane and pump Na^+ out of the cell in exchange for K^+.[35] The most abundant and ubiquitous calpains (μ-calpain and m-calpain, the "conventional" calpains) require Ca^{2+} concentrations that are much higher (10 μm ~ 1 mm) than the physiological intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$, ~100 nm) making them almost inactive in the cytosol.[6, 36] On the other hand, skeletal muscle-specific calpain, p94/calpain 3[8, 37], is unique in that when generated by *in vitro* translation, it undergoes very rapid ($t½$ < 10 min) autolytic degradation even in the absence of Ca^{2+}.[38] These findings indicate that the physiological intracellular ionic environment is sufficient for p94 to be activated, unlike conventional calpains.

Recent research involving calpain 3 protease-inactive knock-in mice have demonstrated that calpain 3 appears to play a role in the Ca2+-efflux from the sarcoplasmatic reticulum in a way that does not involve the protease function of calpain 3, this may explain why dysfunctional calpain 3 leads to muscle weakness.[39] As already mentioned, Calpain 3 is anchored to the giant structural/scaffold protein titin in a stable and inactive manner, to keep it from degrading itself autolytically. For that very same reason, the substrates of calpain 3 is believed to be in close proximity, possibly bound to

other parts of the sarcomeres. It is thought that calpain 3 is inactive most of the time, only to be activated and redistributed when sarcomeres are exercised beyond a threshold, leading to interaction with a number of proteins e.g. myosin light chain 1, suggesting a role for calpain 3 in sarcomere remodeling.[33, 40, 41] Another important in vivo calpain 3 substrates that has been described is AHNAK, a very large protein involved in subsarcolemmal cytostructure and part of the dysferlin membrane repair complex, requires calpain 3 for it to be cleaved and the membrane repair to proceed.[42] Hence, it is a key component in the repair of the wear and tear of skeletal muscle tissue. The search for calpain 3 substrates using cleavage site recognition have lead to a number of potential targets, one being Protein Inhibitor of Activated Stats 3 (PIAS3), an ubiquitously expressed E3 SUMO ligase implicated in many signaling pathways by modifying the localization and role in transcriptional regulation of transcription factors.[43, 44]

In a recent study, it was estimated that about one-third of the LGMD2A biopsies had normal levels of CAPN3 proteolytic activity suggesting that CAPN3 serves other physiological roles besides that of a protease.[47] It has been demonstrated that at least some mutations which do not affect proteolytic activity, decrease the ability of CAPN3 to interact with titin, an established CAPN3-binding partner.[48]

Due to the absence of defined mutational hot spots and presence of mutations in more than one gene, genetic analysis of calpain-3 is not straightforward.[2, 49]

CHAPTER 3

MATERIALS AND METHODS

SAMPLE COLLECTION

3 mL blood samples were collected from three diseased and two normal members of the same family (six siblings) by the method of venipuncture from left/right arm with the use of sterile disposable needle with the help of a Path Lab technician in Solan, Himachal Pradesh.

The blood samples were immediately mixed with 15 µL Na_2EDTA (2.5 mM concentration) to prevent coagulation and transported to the laboratory in cold chain condition.

Pro-formas from 5 volunteers were collected; a copy is attached hereby:

PERFORMA FOR SAMPLE COLLECTION

Sample No.: Date of Collection: Volume of Sample:

Name:

Age:

Sex:

Address:

Telephone Number:

Tests already carried out:

I have been informed that one special blood investigation has to be sent which may or may not benefit me.

Signature

DNA ISOLATION

Preparation of Reagents

- **Tris (hydroxymethyl) aminomethane-chloride (Tris-Cl) (1 M; pH 8.0)**: 75 mL of sterile MQ water was used to dissolve 12.11 gm of Tris base, pH was set to 8.0 with 1 N HCl and the final volume was made 100 mL with MQ water. The solution was filtered through Whatmann filter paper and strored in a sterile tight screw capped reagent bottle.
- **Tris-Cl (1 M; pH 7.3)**: As prepared above, 12.11 gm of Tris base was dissolved in 75 mL of sterile MQ water and the pH was set to 7.3 with 1 N HCl. The final volume of the solution was made 100 mL with MQ water. The solution was filtered and stored in a sterile tight screw capped reagent bottle.
- **Ammonium chloride (NH_4Cl) (1 M)**: Ammonium chloride (5.35 gm) was dissolved in 80 mL of MQ water and final volume of the solution was made 100 mL.
- **Di-sodium ethylene diamine tetra acetate (Na_2EDTA) (0.5 M; pH 8.0)**: Added 18.61 gm of EDTA salt to 50 mL of MQ water in a 250 mL flask and placed it on a magnetic stirrer. Simultaneously, supplemented the solution with 10 M NaOH dropwise, until the pH was reached at 8.0. Allowed the salt to dissolve and then made the final volume of the solution 100 mL with MQ water.
- **Red Blood Cell (RBC) Lysis buffer (10 mM Tris, pH 8.0; EDTA, 1 mM; NH_4Cl, 125 mM; pH 8.0)**: 10 mL Tris (1 M; pH 8.0), 2 mL of EDTA (0.5 M) and 125 mL of NH_4Cl (1 M) were mixed in MQ water to obtain a final volume of 1000 mL RBC lysis buffer.
- **Tris-EDTA (TE) buffer (Tris, 10 mM; EDTA, 1 mM, pH 8.0)**: TE buffer was prepared by mixing 10 mL Tris-Cl (1 M) and 2 mL of EDTA (0.5 M) in 700 mL MQ water and the final volume was made 1000 mL.

- **Tris-EDTA (TE) buffer (Tris, 10 mM; EDTA, 1 mM, pH 7.3)**: As above, 10 mL Tris-Cl (1 M; pH .3) and 2 mL of EDTA (0.5 M) were mixed in MQ water to obtain a final volume of 1000 mL.
- **Sodium dodecyl Sulphate (SDS) 10%**: Dissolved 10 gm of SDS salt in 70 mL of warm MQ water and the final volume was adjusted to 100 mL.
- **Ammonium acetate (7.5 M)**: Dissolved 28.9 gm of ammonium acetate salt in 20 mL of MQ water and the final volume was adjusted to 50 mL.
- **Chilled dehydrated ethyl alcohol**: Undiluted dehydrated ethyl alcohol stored in -20°C deep freezer.
- **Ethanol (70%)**: 70 mL of dehydrated ethanol was added to 30 mL of sterile MQ water to obtain a final volume of 100 mL.

Protocol (Miller et al. 1988)

a. 15 µL Na_2EDTA for 3 mL blood sample (2.5 mM concentration)
b. To 300 µL of blood samples, added RBC lysis buffer (three times the volume of blood sample taken) and kept for incubation on a rocker, to permit perpetual shaking at room temperature (RT) until the RBCs completely lysed.
c. Centrifuged the solution at 13,000 rpm for 1 minute to obtain a creamish white WBC pellet.
d. The supernatant was discarded and the WBC pellet was thoroughly suspended in 300 µL TE buffer (pH 8.0), using a vortexing machine. Thereafter, 20 µL of 10% SDS solution (final concentration (F_C=0.62%) was added to the above solution and the mixture was incubated at 56°C for 30 minutes on a dry-bath.
e. Subsequently, added 150 µL of 7.5 mM ammonium acetate (F_C=2.4 M) and mixed vigorously for about 1 minute per sample, on a vortexer. Centrifuged the mixture at 13,000 rpm, at RT, for 15 minutes, thereby resulting in separation of the precipitated proteins as a pellet.

f. The clear supernatant was transferred to a fresh sterile micro-centrifuge tube. To this added chilled absolute ethyl alcohol (twice the volume of clear supernatant). The tube was gently rocked a couple of times to allow precipitation of genomic DNA.

g. The genomic DNA precipitates were centrifuged at 13,000 rpm for 10 min to pellet at the bottom of the tube. The latter were subsequently washed in 150 µL of 70% ethanol and air dried at RT for about 10-15 minutes.

h. 100 µL of TE buffer (pH 7.3) was used to dissolve the dried DNA pellet by incubating at 65°C for 10 minutes. The dissolved DNA was finally stored at -20°C till further use.

AMPLIFICATION USING PCR (POLYMERASE CHAIN REACTION)

The **polymerase chain reaction (PCR)** is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR

methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps. The first step is to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. The second step is to lower the temperature. Then each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

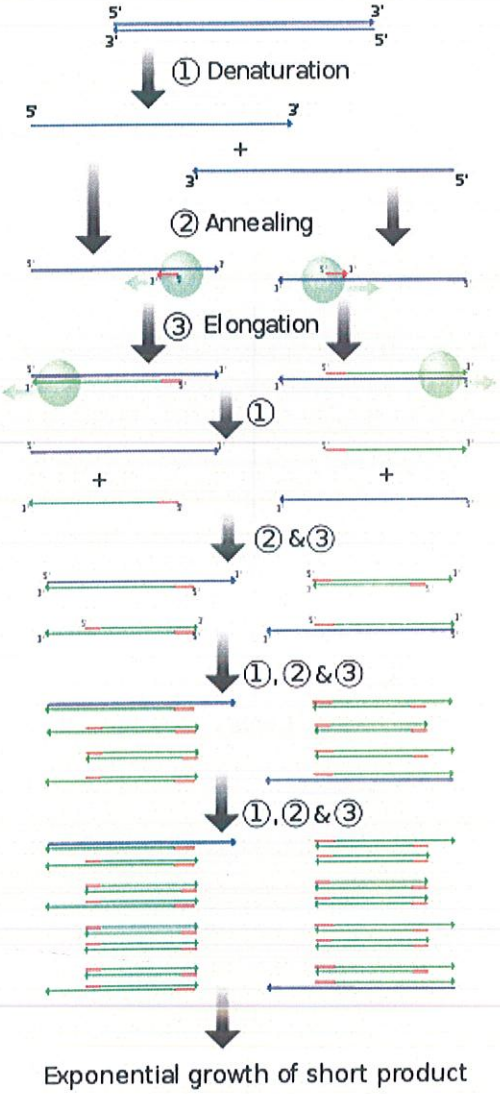


Figure 4

Primer Design

Here primers against exon 13 of CAPN3 were obtained. The primers used were ordered from IDT (Integrated DNA Technologies):

CAPN3-13F	5'-TGT GGC AGG ACA GGA CGT TC-3'
CAPN3-13R	5'-TTC AAC CTC TGG AGT GGG CC-3'

Table 2

Reaction Mixture

The reaction mixture was prepared as follows:

Component	**Volume (µL)**
DNA	4
Primer 1	0.6
Primer 2	0.6
Master Mix	12.5
Distilled Water	7.3
Total Volume	**25**

Table 3

Conditions

We followed the following conditions for PCR:

Step	Temperature (°C)	Time	
Initial Denaturation	94	3 min	
Denaturation	94	30 sec	35 cycles
Annealing	56	30 sec	
Extension	72	30 sec	
Final Extension	72	5 min	

Table 4

SSCP (Single-strand Conformation Polymorphism)

Single-strand conformation polymorphism (SSCP), or single-strand *chain* polymorphism, is defined as conformational difference of single-stranded nucleotide sequences of identical length as induced by differences in the sequences under certain experimental conditions. This property allows distinguishing the sequences by means of gel electrophoresis, which separates the different conformations.

A single nucleotide change in a particular sequence, as seen in a double-stranded DNA, cannot be distinguished by electrophoresis, because the physical properties of the double strands are almost identical for both alleles. After denaturation, single-stranded DNA undergoes a 3-dimensional folding and may assume a unique conformational state based on its DNA sequence. The difference in shape between two single-stranded DNA strands with different sequences can cause them to migrate differently on an electrophoresis gel, even though the number of nucleotides is the same, which is, in fact, an application of SSCP.

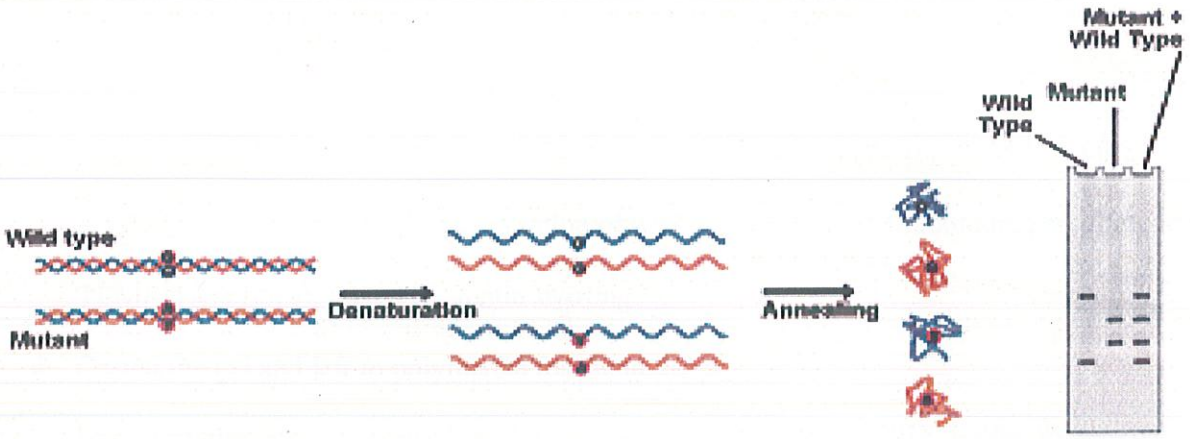


Figure 5 www.nationaldiagnostics.com

Protocol for SSCP

Sample Preparation

1. 7µL of amplified DNA was loaded per well. Aliquot proper amount of sample into separated tubes and added an equal volume of 2X SSCP gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA).
2. Prior to loading, denatured the samples at 95°C for 5 min and then placed on ice.

PAGE (Poly Acrylamide Gel Electophoresis)

PAGE was carried out in Biorad Protease Mini Gel. (Figure below)

1. Prepared 6 % polyacrylamide gels (37.5:1 acrylamide to bisacrylamide) with or without 5% glycerol.

10X TBE gel buffer	-	2 mL
29.1% acrylamide:bisacrylamide solution	-	4 mL
10% ammonium persulfate	-	100 µL
Water	-	13.88 mL
TEMED	-	20 µL

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2. De-gassed the acrylamide solution under vacuum until bubbles no longer appeared (5- 10 min).
3. Added 12 µL 10% ammonium persulfate (freshly made up) and 12 µL TEMED to the acrylamide solution (a final concentration of 0.09% (v/v) each of ammonium persulfate and TEMED solutions), mixed by gentle swirling.
4. Casted the gel and left to polymerise for about 2-3 h.
5. Removed the comb and washed the sample wells with 0.5 x TBE buffer to remove the unpolymerised polyacrylamide.
6. Ran the gel in 0.5X TAE buffer that had been pre-cooled to 10°C. Placed the gel tank in a tray filled with ice. Set the voltage to 150 V and ran the gel for about 3.5 h. The temperature at the end of the run was about 17°C.

SILVER STAINING

Silver staining is the use of silver to selectively alter the appearance of a target in microscopy of histological sections; in temperature gradient gel electrophoresis; and in polyacrylamide gels.

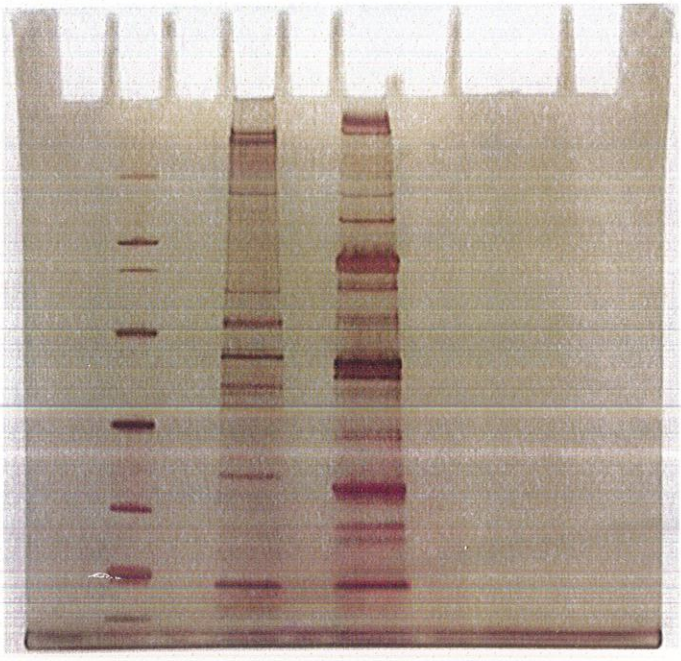


Figure 6

Protocol

1. Disassembled gel apparatus carefully, separating the glass plates.
2. Placed glass plate with bound gel onto plastic tray.
3. Applied 100 mL fixing solution (10% ethanol, 1% acetic acid) and shook gently for 10 min.
4. Washed gel with distilled H_2O for 1 min.
5. Pretreated gel (oxidize) with 100 mL of 1.5% nitric acid for 3 min, shaking gently.
6. Rinsed gel with 100 mL distilled H_2O for 1 min.
7. Impregnated gel with 100 mL of 0.2% AgNO3 solution for 20 min, shaking gently.
8. Rinsed gel with 100 mL distilled H_2O for 30 s, twice.
9. Developed gel by applying, initially 100 mL of cold (ca. 12°C) developing solution (30 g L^{-1} Na_2CO_3; 0.54 mL 37% formaldehyde $liter^{-1}$) and gently shook until the solution became dark. Replaced the solution with 100 mL of fresh cold solution, for 4-7 min, until the bands appeared with desirable intensity (ca. 5 min). Removed developing solution.
10. Stopped developing reaction by adding 100 mL of 5% acetic acid for 5 min.
11. Washed gel in distilled water.
12. Air dried and photographed.

CHAPTER 4

RESULTS AND DISCUSSION

NOTE: Sample nos. 1, 2 and 3 were of the diseased, and, 4 and 5 were of the normal siblings.

DNA ISOLATION

DNA was isolated from the blood samples collected from 5 siblings. The isolation was then checked by running an Agarose Gel Electrophoresis. The result was as seen below:

1 2 3 4 5 6 7 8 9 10 11 12 13

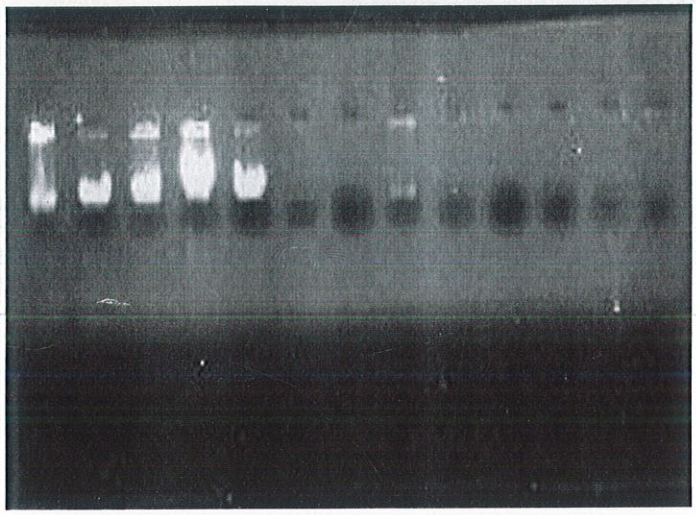
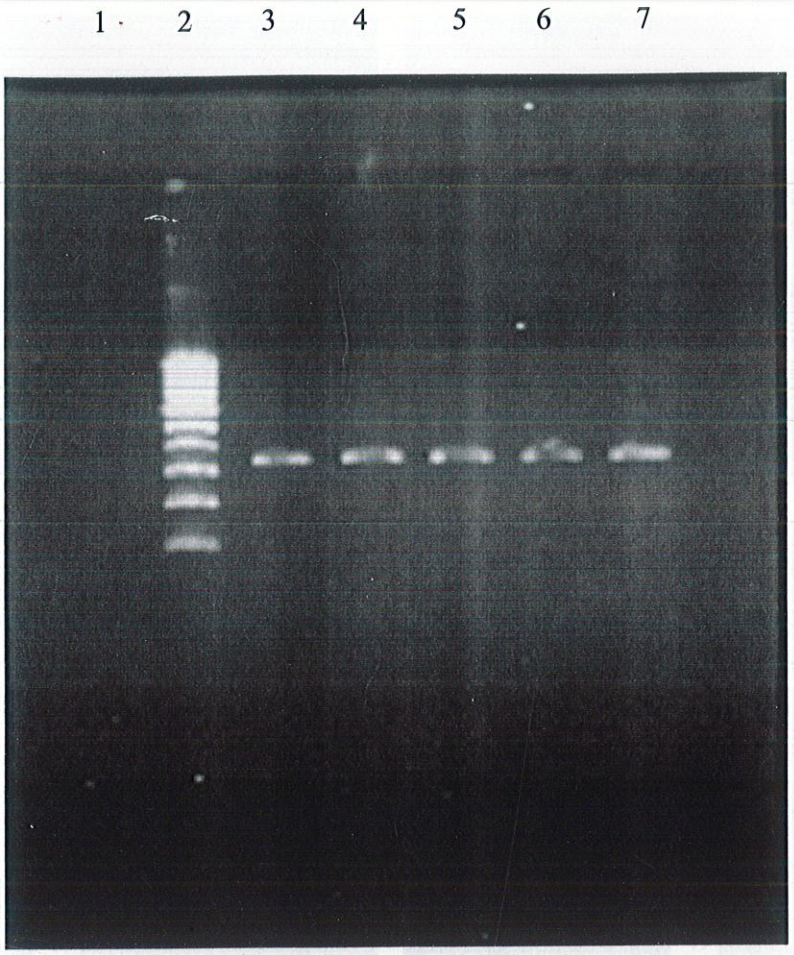


Figure. Agarose Gel Electrophoresis of isolated DNA

Lane 1: Sample 1; **Lane 2**: Sample 2; **Lane 3**: Sample 3; **Lane 4**: Sample 4; **Lane 5**: Sample 5

PCR AMPLIFICATION

The isolated DNA was then amplified using the conditions mentioned in the 'Materials and Methods' section. This amplified product was then checked by running another Agarose Gel Electrophoresis. The result was as following:



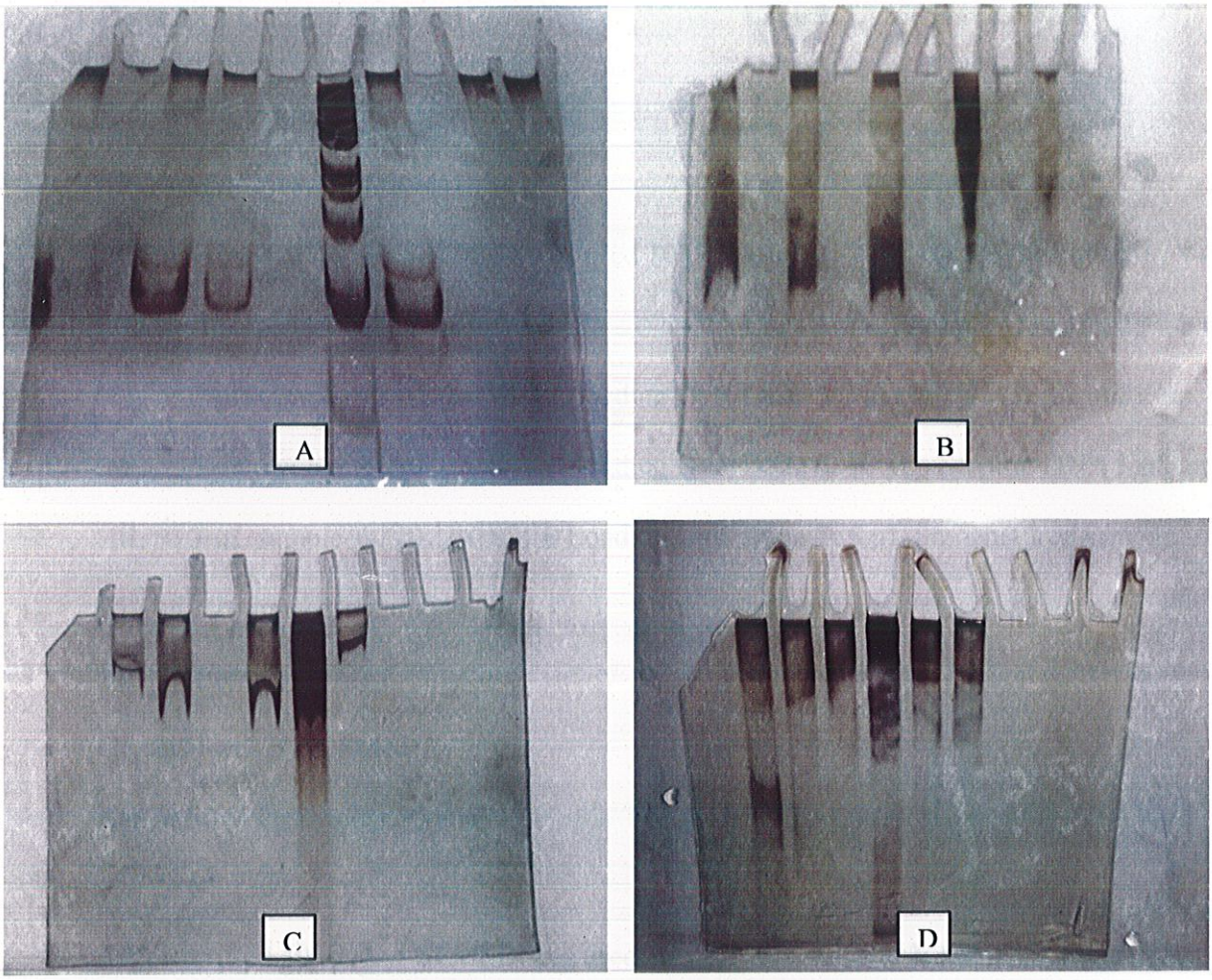
Agarose gel electrophoresis of PCR products

Lane 2: DNA Step-Up Ladder (100 bp); **Lane 3**: Sample 1 (Patient); **Lane 4**: Sample 2 (Patient); **Lane 5**: Sample 3 (Patient); **Lane 6**: Sample 4 (Normal); **Lane 7**: Sample 5 (Normal)

The bands of the amplicons formed somewhere around 300 bp. Thus, these sequences are of the amplified exon 13.

SSCP

Before getting the results, SSCP was needed to be standardized. We carried out this experiment six times, and performed silver staining with all of them. Unfortunately, the bands were seen only in four of them. The following are the pictures of those four run SSCPs with the alterations done each time in order to standardize.



A. 6% PAGE (30% Acrylamide)

APS and TEMED were used 12 µL each

7 µL sample + 7 µL 2X SSCP Loading Dye

95°C for 5 min- denaturation

TAE buffer used

Run at 150 V for 3.5 hours.

B. 8% PAGE (40% Acrylamide)

APS and TEMED were used 12 µL each

5 µL sample + 5 µL 2X SSCP Loading Dye

95°C for 5 min- denaturation

TBE buffer used

Lane 1: Sample no. 1 (denatured)

Lanes 2, 4, 6, 8, 10: Blank

Lane 3: Sample no. 2 (denatured)

Lane 5: Sample no. 3 (denatured)

Lane 7: 250 bp step-up DNA Ladder

Glycerol was added to the gel

Run at 150 V for 2 hours 45 min

Lane 9: Un-denatured DNA

C. 8% PAGE (30% Acrylamide)

APS was used 50 µL and TEMED was used 10 µL

Different amounts of samples were used:

i. 3 µL sample + 3 µL 2X SSCP Loading Dye
ii. 5 µL sample + 5 µL 2X SSCP Loading Dye
iii. 7 µL sample + 7 µL 2X SSCP Loading Dye

95°C for 10 min- denaturation

TBE buffer used

Glycerol was NOT added

Run at 50 V for 4 hours 30 min

Lanes 1, 4, 8, 9, 10: Blank

Lane 2: 3 µL sample (denatured)

Lane 3:5 µL sample (denatured)

Lane 5:7 µL sample (denatured)

Lane 6: 100 bp Step-up DNA Ladder

Lane 7: 5 µL un-denatured sample

D. 6% PAGE (30% Acrylamide)

APS was used 50 µL and TEMED was used 10 µL

4 µL sample + 4 µL 2X SSCP Loading Dye

95°C for 10 min- denaturation

TBE buffer used

Glycerol was NOT added

Run at 100 V for 2 hours 45 min

Lane 1: Cut

Lane 2: Sample 1 (denatured)

Lane 3: Sample 2 (denatured)

Lane 4: Sample 3 (denatured)

Lane 5: DNA ladder

Lane 6: Sample 4 (denatured)

Lane 7: Sample 5 (denatured)

In the gel D, we can faintly see three bands in all the samples, proving that our samples had indeed been denatured properly and that the problem was with gel running protocol and not any steps prior to that.

CHAPTER 5

CONCLUSION

This study was essentially taken up to find out the mutations that occur in the exon 13 of CAPN3 gene, that cause Calpainopathy. This is a genetic disease and the blood samples were collected from siblings to prove that even though majority of the genome of these siblings is same, there are considerable differences which cause this disease. If the SSCP results would have come out right, we would have been able to prove that both the genomes, of diseased as well as normal, have mutational differences. Next, we would have sent if for sequencing to see the polymorphisms at nucleotide base level. However, because most of our time was utilized in standardizing the SSCP protocol, we could not complete the experiment to the expected level. We hope to continue the same topic in our final year of 5 Year Dual Degree Programme B. Tech - M. Tech in Biotechnology.

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CERTIFICATION:

I, the undersigned certify that to the best of knowledge and belief, The Resume correctly describes my qualification and myself.

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CERTIFICATION:

I, the undersigned certify that to the best of knowledge and belief, The Resume correctly describes my qualification and myself.

SP0913131

Date: 27th May 2013

Place: JUIT, Waknaghat

Lavanya Jain