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Genetic Studies and Computational Analysis of Hereditary Limb Anomalies and Muscular Dystrophy

Researcher

Sumaira Nishat.

Reg #: 08/FBAS/MSBI/F07

Supervisors

Dr. Naveeda Riaz.

Mr. M. Ismail.

Department of Bioinformatics

Faculty of Basic & Applied Sciences

International Islamic University Islamabad

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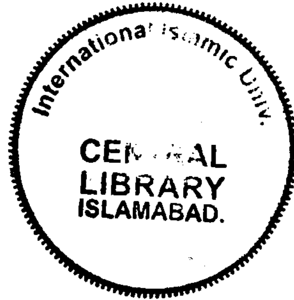

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Genetic Studies and Computational Analysis of Hereditary Limb Anomalies and Muscular Dystrophy

Sumaira Nishat
Reg #: 08/FBAS/MSBI/F07

Submitted in partial fulfillment of the requirements for the Masters of Sciences in Bioinformatics at the Department of Bioinformatics, Faculty of Basic and Applied Sciences, International Islamic University Islamabad.

Supervisors: Dr. Naveeda Riaz.
Mr. M. Ismail.

27th August, 2009

Verily, when He intends a thing, His Command is, "be", and it is!

(Ya-Sin: 82)

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Sumaira Nishat

Dedicated to my sweet parents

Mr. Nishat Ahmed & Mrs. Fazeelat Anwar

CERTIFICATE

Title of Thesis: Genetic Studies and Computational Analysis of Hereditary Limb Anomalies and Muscular Dystrophy

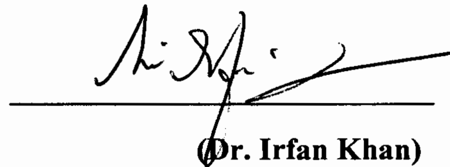
Name of Student: Sumaira Nishat

Registration No: 08/FBAS/MSBI/F07

Accepted by the Department of Bioinformatics, Faculty of Basic and Applied Sciences, International Islamic University Islamabad, in partial fulfillment of the requirements for the Master of Sciences in Bioinformatics.

Viva Voce Committee

Chairman



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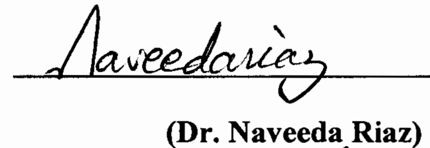
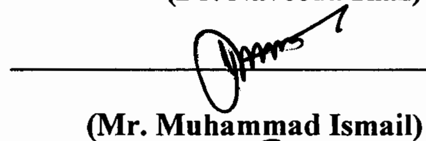


(Dr. Naveeda Riaz)

External Supervisor



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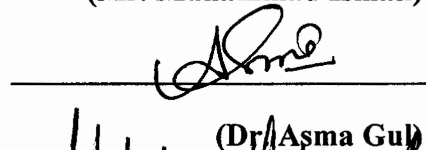

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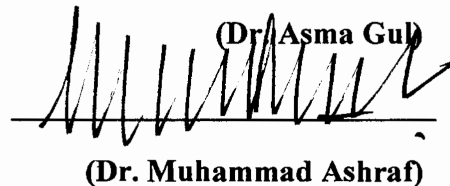

(Dr. Muhammad Ashraf)

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ABSTRACT

Genetic studies have contributed much in establishing the linkage association for genetic disorders and have been employed widely in identification of defective gene and affective drug targets against it but this traditional approach is limited as it generates large data sets. This obstacle is overcome by introducing Bioinformatics approaches. For the present study, four families with Limb anomalies and muscular dystrophies have been ascertained from different regions of Pakistan.

Syndactyly, a congenital malformation of limbs, involves the fusion of two or more fingers and/or toes. Eight types of syndactyly have been described yet. All types except Cenani-Lenz type, show autosomal dominant mode of inheritance.

In Family A, affected individuals exhibited typical feature of Syndactyly type I i.e. incomplete fusion of third and fourth fingers. The linkage data obtained form scan of the five chromosomal regions failed to define a region harboring a causative gene for Syndactyly.

In Postaxial Polydactyly one or more extra finger and/or toe are present on the ulnar or fibular side of the hands and feet, respectively. It is inherited as an autosomal dominant trait, while autosomal recessive trait has also been reported. Currently, four loci have been described for Postaxial Polydactyly in humans and only two responsible genes i.e. GLI3 and HOXA have been identified yet at Postaxial Polydactyly-A1.

Postaxial Polydactyly observed in Family B clinically fits Postaxial Polydactyly-A3. The disorder is consistent with an autosomal dominant trait, since patients of both sexes were equally affected. Linkage analysis demonstrated that Postaxial Polydactyly-A3 locus was assigned to chromosome 19p13.2-13.2.

Candidate gene identification might add up our knowledge of limb development, particularly developmental stages of digit identity, number and separation. Computational tools i.e. G2D, SUSPECT, PROSPECTR, GeneWanderer, GeneDistiller and PosMed have been employed to gather a subset of genes that are positionally linked to and could be causing Postaxial Polydactyly-A3. Following genes have been identified for their candidature: Zinc finger containing genes, KLF1, ECSIT homolog, JUND, RAD23A, TNPO2 and ASNA-I.

Different types of Muscular Dystrophies are defined by the disease course, age of onset and severity. Family C and D shows typical feature of Muscular Dystrophy. The linkage data obtained form scan of the associated chromosomal regions failed to define a region harboring a causative gene for Muscular Dystrophy.

Protection against oxidative stress is possible by consumption of antioxidants. It has been proved that oxidative stress is a major cause of Muscular Dystrophies. Triterpene antioxidants, including Ganoderic acids A, B, C and D; Ganoderol A and B; and Ganodermanontriol found in Reishi extracts, have been analyzed computationally and compared with supplements i.e. Creatine and Coenzyme Q10, being used for treating muscular dystrophy. Docking analysis has favored the consumption of Ganoderic acid A and Ganoderic Acid B for all Muscular Dystrophies. Also Docking results show that Creatine is better supplement than coenzyme Q10. I believe that the docking results presented in this study will prove to be a tremendous help for families suffering with Muscular Dystrophies.

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All praises are due to Almighty ALLAH , the Most Merciful and compassionate and His Holy Prophet Muhammad (Peace be upon him) , the most perfect and exalted among and of ever born on the surface of earth, who is for ever a torch of guidance and knowledge for humanity as a whole.

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Road is long and life is short, may ALLAH give me the valor and opportunity to discover and face the truth (Ameen).

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LIST OF ACRONYMS

ACD	Acid Citrate Dextrose
BMD	Muscular Dystrophy, Becker Type
CAPN3	Proteolytic enzyme Calpain-3
CAV3	Caveolin-3
CHLC	Cooperative Human Linkage Center
CLS	Cenani-Lenz syndrome
COL6	Collagen type VI
CMD	Congenital Muscular Dystrophies
DAG	Alpha-Dystroglycan
DigiCGA	Digital Candidate Gene Approach
DM	Dystrophia Myotonica
DMAT	Myopathy, Distal, With Anterior Tibial Onset
DMD	Muscular Dystrophy, Duchenne Type
DMPK	Dystrophia Myotonica Protein Kinase
DYSF	Dysferlin
ECSIT	Evolutionarily Conserved Signaling Intermediate in Toll pathway
EDMD	Emery-Dreifuss Muscular Dystrophy, X-Linked
FKTN	Fukutin
FCMD	Fukuyama Congenital Muscular Dystrophy
FBLN	Fibulin-1
FKRP	Fukutin-Related Protein
FSHMD	Facioscapulohumeral muscular dystrophy
GLI3	Gli-Kruppel Family Member 3
GLUE	Genetic Linkage User Environment
HOXD13	Homeobox D13
JUND	JUN D proto-oncogene
KLF1	Kruppel like factor 1
LAMA2	Laminin alpha-2
LARGE	Acetylglucosaminyltransferase-Like Protein
LMNA	Laminin A/C
LGMD	Limb-Girdle Muscular Dystrophy
MEB	Muscle-Eye-Brain Disease

MD	Muscular Dystrophy
MM	Miyoshi Myopathy
MYH6	Myosin, Heavy Chain 6, Cardiac Muscle, Alpha
ODD	Oculodentodigital Dysplasia
OMIM	Online Mendelian Inheritance in Man
POMGNT1	O-mannose beta-1, 2-N-acetylglucosaminyltransferase
OPMD	Oculopharyngeal Muscular Dystrophy
PCR	Polymerase Chain Reaction
PAP	Postaxial Polydactyly
POMT1	Protein O-Mannosyltransferase-1
RSMD1	Rigid Spine Muscular Dystrophy 1
SEPN1	Selenoprotein N, 1
SGCA	Alpha-Sarcoglycan
SGCB	Beta-Sarcoglycan
SGCD	Sarcoglycan-Delta
SGCG	Gamma-Sarcoglycan
SPD	Synpolydactyly
T2D	Type 2 Diabetes
TCAP	Telethonin
TRIM32	Tripartite Motif-Containing Protein-32
TTID	Myotilin
TTN	Titin
UCMD	Ullrich Congenital Muscular Dystrophy
WDM	Welander Distal Myopathy
WWS	Walker-Warburg Syndrome
ZD1	Zygodactyly 1
ZN	Zinc Finger

INTRODUCTION

In humans, diseases have diverse causes which can be classified into two broad categories: Diseases caused by microorganisms and Genetic disorder. The diseases which are caused by some microorganisms can spread from one person to another but are not inheritable (except some viral diseases), While genetic diseases, result from single gene alterations, chromosomal abnormalities or multi-factoarial errors, are heritable and may become lethal.

Genetic studies have contributed much in establishing the linkage association for genetic disorders. They have been employed widely in identification of defective gene and its normal biological role but this traditional approach is limited as it generate large candidate gene sets and also it depends on the previous knowledge about physiological, biochemical or functional aspects of possible candidates. This barrier is breached by introducing computational approaches to identify candidate diseases genes. Same way, several drugs in the past were either developed experimentally or discovered accidently but now by using the power of Bioinformatics, it is possible to generate wealth of information that can be used to discover and select suitable targets for new drugs. For the present study, two limb anomalies i.e. Syndactyly and polydactyly, while one muscular disorder i.e. muscular dystrophy has been selected.

CONGENITAL LIMB ANOMALIES

Wide-ranging congenital limb anomalies e.g. Syndactyly and Polydactyly reflect the complication and accuracy of limb development. Identification and characterization of the underlying gene(s) can increase our understanding of normal limb development.

SYNDACTYLY

Non-syndromic Syndactyly is a heterogeneous hereditary condition in which two or more fingers and/or toes are joined together. It is a developmental abnormality during a sixth to eighth weeks of intra-uterine life when normal separation of the fingers does not occur by pre-programmed cell death that normally occurs. It is two times more common in males. The distribution of webbing between the digits varies (Adrian *et al.*, 2005).

Syndactyly has a frequency of 1:2000 in the population and the webbing may be bony or cutaneous. Exogenous Syndactyly (fusion between the more distal portions of the

digits) is caused by mechanical influences during embryogenesis while endogenous Syndactyly can either be a symptom of a syndrome, such as Apert or Oculo-Digital-syndrome, or of an isolated malformation (de Smet *et al.*, 1994).

Classification of Syndactyly

On the basis of an anatomic approach, Non-Syndromic Syndactylies has been subdivided into eight major types (Temtamy and McKusick, 1978; Goldstein *et al.*, 1994).

Syndactyly Type I or Zygodactyly (MIM 185900)

Syndactyly type I is described by complete or partial webbing between the third and fourth fingers, second and third toes may or may not be included. In some cases the webbing between fingers is associated with fusion of the distal phalanges. This is the most common type of Syndactyly which accounts for the majority of isolated syndactylies (Castilla *et al.,* 1980).

Syndactyly type I shows autosomal dominant trait, and the occurrence of skipped generations indicates that penetrance is <100% (Montagu, 1953). The gene for Syndactyly type I has been localized in a large German family to chromosome 2q34-q36 (Bosse *et al.,* 2000). The clinical spectrum of digital malformation in the German family reached from skin fusion between 2nd and 3rd toes to complete webbing between the 2nd to 5th fingers and 1st to 5th toes. Ghadami *et al.,* (2001) reported an Iranian family which was also linked to the same locus on chromosome 2q34-q36.

Malik *et al.*, 2005 predicted genetic heterogeneity in zygodactyly. He localized a new gene for Syndactyly type I on 3p21.31 by genome-wide linkage analysis in a Pakistani family while, in German family, zygodactyly was linked to neither 2q31-q36 nor 3p21.31. He proposed to designate the 3p21.31 locus as zygodactyly 1 (i.e. ZD1).

Synpolydactyly Type II (MIM 18600)

Synpolydactyly (SPD) is characterized as a cutaneous or bony fusion between the middle and ring fingers associated with complete or partial duplication of the ring finger in the web. Duplication of fifth toe in the feet is a usual finding (Temtamy and McKusick, 1978).The more extreme phenotype shows complete soft tissue syndactyly involving both hands and feet. In the hands there is polydactyly of the preaxial,

mesoaxial, and postaxial digits, loss of the normal tubular shape of the carpal, metacarpal, and phalangeal bones (Akarsu *et al.,* 1997).

SPD shows an autosomal dominant mode of inheritance (Sayli *et al.,* 1995). First linkage was reported to chromosome 2q31 in a large Turkish family (Sarfarazi *et al.,* 1995). Polyalanine tract expansion mutations in the Homeobox containing gene Homeobox D13 *(*HOXD13; MIM 142989*)* have been described for SPD (Muragaki *et al.,* 1996). A complex type of SPD was observed in a patient with chromosomal translocation, t(12;22), disrupting the fibulin-1 gene (FBLN; MIM 135820) on chromosome 22q13.3 (Debeer *et al.,* 2002). Kan *et al.,* (2003) also reported mutations of HOXD13 in patients with a variety of limb malformations.
Malik *et al.,* 2006 reported genetic heterogeneity for SPD by making use of a genomic-wide analysis in large Pakistani family and localized a new gene for SPD at chromosome 14qll.2-ql2. Malik *et al.,* (2007) proved that expansion of alanine residues in the HOXD13 polyalanine repeat is not linked with the SPD1 phenotype.

Malik and Grzeschik (2008) showed that typical SPD features can be delineated from minor clinical variants. They proposed to lump all the phenotypic variants, manifesting themselves in SPD families into three categories: (i) typical SPD features, (ii) minor variants, and (iii) unusual phenotypes.

Syndactyly of fingers 4 and 5, Type III (MIM 186100)

It is described by fusion between the fourth and fifth fingers. It has been rdescrbed as a part of Oculodentodigital Dysplasia (ODD; MIM 16420). Gladwin *et al.,* (1997) localized the gene for ODD on chromosome 6q22-q24. They proposed that isolated type III Syndactyly may be encoded by the same gene as ODD syndrome. Boyadjiev *at al.,* (1999) refined the location of ODD gene.

Paznekas *et al.,* (2003) found mutations in Gap Junction Protein, Alpha-1 (GJA1; 121014) gene in families with ODD. Vitiello *et al.,* (2005) identified novel heterozygous missense mutation in the GJA1 gene in an Italian family

Haas-type Syndactyly, Type IV (MIM 186200)

It is described by complete fusion of all fingers in both hands that gives hands a cup-shaped form. Association of polydactyly is also observed. When feet are involved, they usually show complete fusion of all toes (Haas *et al.,* 1940; Gillessen-Kaesbach and Majewski, 1991).

Haas type Syndactyly is a rare phenotype, and there are only four reports available in the literature. The most likely mode of inheritance is autosomal dominant with variable expressivity. Sato *et al.,* (2007) localized gene for type IV Syndactyly to chromosome 7q36 between markers D7S3070 and D7S559.

Sun *et al.,* (2008) reported that Triphalangeal thumb-polysyndactyly syndrome (TPTPS) and Syndactyly type IV are due to genomic duplications involving the limb-specific Sonic Hedgehog (SHH; MIM 600725) protein enhancer.

Syndactyly with Metacarpal and Metatarsal, Type V (MIM 186300)

Syndactyly type V is the presence of an associated fourth and fifth or the third and fourth metacarpal and metatarsal fusion. It is a rare autosomal dominant type with only two reports published so far (Temtamy and McKusick, 1978; Malik *et al.,* 2005; Zhao *et al.,* 2007). Syndactyly with Metacarpal and Metatarsal is reported to be caused either by missense mutation or polyanaline expansion in HOXD13 gene localized in 2q31-q32 (Kemp and Ravn, 1932; Kjaer *et al.,* 2005; Zhao *et al.,* 2007).

Syndactyly Type VI, Mitten syndactyly

It is characterized by webbing of digits 2-5 in both hands and feet. It shows autosomal dominant mode of inheritance (Temtamy and McKusick, 1978). No MIM number has yet been allocated to this phenotype.

Syndactyly Type VII, Cenani-Lenz Syndactyly (MIM 212780)

It is characterized by complete Syndactyly of hands and feet, abnormal phalangs, carpal and metacarpal fusion. This is the only type which shows autosomal recessive mode of inheritance. No linkage has been reported yet for Cenani-Lenz syndactyly (Cenani and Lenz, 1967; Bacchelli *et al.,* 2001; Temtamy *et al.,* 2003).

Syndactyly Type VIII

Syndactyly type VIII is characterized by unilateral or bilateral fusion of metacarpal fourth and fifth. Degree of fusion may range from minimal to complete. An autosomal dominant inheritance was suggested for the isolated forms while X-linked recessive for familial cases (Lonardo *et al.,* 2004). No MIM number has been allocated yet to this phenotype.

POSTAXIAL POLYDACTYLY (PAP; MIM 174200)

Postaxial Polydactyly is described as "presence of one or more extra finger and/or toe on the ulnar or fibular side of the hands and feet, respectively". Depending upon analysis of various pedigrees with PAP, Phenotypically It is subdivided into two types. In Postaxial Polydactyly type A (PAP-A), the extra digit is coherent with the fifth or extra metacarpal/metatarsal, well developed, and is usually functional. In Postaxial Polydactyly type B (PAP-B), the extra digit is not well formed and mostly present as a skin tag (Temtamy and McKusick 1969).

Among the chromosomal abnormalities, postaxial polydactyly occurs with trisomy 13, in which about 75% of cases show this feature. It shows autosomal dominant inheritance. Sometimes PAP-A and PAP-B appear in the same generation (Sverdrup 1922; Odiorne 1943; Ventruto *et al.,* 1980; Kucheria *et al.,* 1981). Postaxial polydactyly has also been reported as an autosomal recessive trait (Mohan, 1969; Cantu *et al.,* 1974; Mollica *et al.,* 1978).

Currently, there are four loci for Postaxial Polydactyly in humans. A frameshift mutation in the Gli-Kruppel Family Member 3 (GLI3; 165240) gene was found by Radhakrishna *et al.,* (1997) in a five generation Indian kindred with Autosomal Dominant Postaxial Polydactyly, Type A1 (PAP-A1; MIM 174200) linked to Chromosome 7p15-q11.23. Furniss *et al.,* (2007) reported a patient with postaxial polydactyly type B of the hands having heterozygous mutation in the GLI3 gene. Postaxial Polydactyly, Type A2 (PAP-A2; MIM 602085) was mapped to chromosome 13q21-q32 after performing a linkage study in a family with postaxial polydactyly type A (PAPA) (Akarsu *et al.,* 1997). Zhao *et al.,* (2002) reported locus for Postaxial Polydactyly, Type A3 (PAP-A3; MIM 607324) on chromosome 19p13.1-13.2 in a large 6-generation Chinese family with a combination of Type A and B (PAP-A/B). Fourth locus for Postaxial Polydactyly, Type A4 (PAP-A4; MIM 608562) was identified by Galjaard *et al.,* (2003) on chromosome 7q21-q34 in a Dutch family with PAP-A/B and incomplete cutaneous Syndactyly.

MUSCULAR DYSTROPHY (MD)

MDs are hereditary diseases of muscular system. They are characterized by progressive degeneration and weakness of body muscles. Every muscle has different rate of progression (Emery, 2008). It affects the voluntary muscles. Over time the

weakness gets worst and different muscles are affected. In some cases the heart and lungs can be affected. Wrong or missing genetic information in the body's cells may lead to MD. The person's body is unable to make the proteins needed to make and retain the healthy muscles (Johanson, 2008).

Classification of Muscular Dystrophy

Following types of MDs have been described yet.

Muscular Dystrophy, Becker Type (BMD; MIM 300376)

This type of muscular dystrophy is characterized by Priximal muscle weakness, loss of ambulation and mental impairment. Age of onset is around 12 years while death is usually in fourth or fifth decade (Emery, 2002). It shows X-linked inheritance and was found to be linked with chromosomal region Xp21.2 (Becker, 1957; Kingston *et al.,* 1983, 1984, Brown *et al.,* 1985; Fadda *et al.,* 1985; Roncuzzi *et al.,* 1985). BMD is caused by mutation in dystrophin (DMD; MIM 300377) encoding gene (Passos-Bueni *et al.,* 1994; Arikawa-Hirasawa *et al.,* 1995; Yagi *et al.,* 2003; Tuffery-Giraud *et al.,* 2005).

Congenital Muscular Dystrophy (CMD)

CMDs are autosomal recessive disorders and are characterized by muscle weakness, contractures and in some casese brain defects are also observed (Pegoraro *et al.,* 2000).

Muscular Dystrophy, Congenital Merosin-Deficient, 1A (MDC1A; MIM 607855)

Hillaire *et al.,* (1994) demonstrated that MDC1A is linked to chromosomal region of 6q2. Primary deficiency of the laminin alpha-2 gene (LAMA2; MIM 156225) because of mutations in this gene is described as major cause of MDC1A. In some cases it was found to be associated with extensive brain abnormalities (Pegoraro *et al.,* 2000; Jones *et al.,* 2001; Tezak *et al.,* 2003; Di Blasi *et al.,* 2005; Oliveira *et al.,* 2008).

Muscular Dystrophy, Congenital, 1B (MDC1B; MIM 604801)

This form of CMD is characterized by proximal muscle weakness, increase in muscle size, early respiratory failure and muscle weakness. Pattern of inheritance is autosomal recessive. Affected individuals were demonstrated to have a secondary

deficiency of LAMA2 in muscle (Muntoni *et al.,* 1998). Brockington *et al.,* (2000) performed genomewide linkage analysis in two different families with MDC1B and reported linkage to chromosome 1q42.

Muscular Dystrophy, Congenital, 1C (MDC1C; MIM 606612)

In this unique form of CMD patients had onset at birth and are characterized by increase in size of lower limb muscles, failure of ambulation, facial weakness (Brockington *et al.,* 2001; Mercuri *et al.,* 2003; Topaloglu *et al.,* 2003; Louhichi *et al.,* 2004). Mutation in the gene encoding the fukutin related protein (FKRP; MIM 606596), located on chromosome 19q13.3 are reported as major cause of MDC1C (Brockington *et al.,* 2001; Louhichi *et al.,* 2004; MacLeod *et al.,* 2007).

*Muscular Dystrophy, Congenital, Type 1D (*MDC1D*; MIM 608840)*

This type of CMD is characterized by Mental retardation and congenital muscular Dystrophy. Mutation in the gene encoding acetylglucosaminyltransferase-like protein (LARGE; MIM 603590) is major cause of MDCID. LARGE is located on chromosomal region 22q12.3-q13.1, as MDC1D (Longman *et al.,* 2003).

Rigid Spine Muscular Dystrophy 1 (RSMD1; MIM 602771)

It shows autosomal recessive mode of inheritace with variable penetrance and sex-linked expression (Ferreiro *et al.,* 2002). First linkage was reported to chromosome 1p36-p35 in the large consanguineous family and found evidence of linkage associated with the Selenoprotein N, 1 (SEPN1; MIM 606210) gene. Mutations in the SEPN1 gene resulting in RSMD1 were also identified (Moghadaszadeh *et al.,* 1998; Ferreiro *et al.,* 2002, 2004; Flanigan *et al.,* 2000).

Ullrich Congenital Muscular Dystrophy (UCMD; MIM 254090)

UCMD is characterized by proximal contractures at birth or in early childhood, limitation of motion in axial and proximal joints, distal hyperextensibility and hip dislocation (Mercuri *et al.,* 2002). Demir *at al.,* (2002) described that UCMD is an autosomal recessive disorder and shows linkage to 2q37. Mutations in any of three genes encoding the subunits of Collagen type VI (COL6A1, MIM 120220; COL6A2, MIM 120240; and COL6A3, MIM 120250 (Pan *et al.,* 2003; Baker *et al.,* 2005; Giusti *et al.,* 2005; Lampe *et al.,* 2005; Angelin *et al.,* 2007; Kawahara *et al.,* 2007).

Walker-Warburg Syndrome (WWS; MIM 236670)

It is illustrated as low muscle tone at birth, inability to walk, contractures, absent speech and mental retardation (Villanova *et al.,* 2000). Van Reeuwijk *et al.,* (2006) identified compound heterozygous mutations in the protein O-mannosyltransferase-1 (POMT1; MIM 607423) gene in patients from two families reported by Villanova *et al.,* (2000).

Fukuyama Congenital Muscular Dystrophy (FCMD; MIM 253800)

CMD is characterized by muscle weakness and low muscle tone by childhood, unablility to walk without support, mental retardation, seizures and brain malformations. It shows autosomal recessive inheritance (Fukuyama *et al.,* 1960). Gene for FCMD was localized on chromosome 9q31-q33 (Toda *et al.,* 1996). Mutation in the gene encoding fukutin (FKTN; MIM 607440) may lead to FCMD (Kobayashi *et al*., 1998; Kondo-Iida *at al*., 1999).

Muscle-Eye-Brain Disease (MEB; MIM 253280)

It is described as CMD with severe inherited myopia and mental retardation (Raitta *et al.,* 1978). Cormand *et al.,* (1999) reported linkage for MEB gene on chromosome1p34-p32. O-Mannose beta-1, 2-N-Acetylglucosaminyltransferase (POMGNT1; MIM 606822) gene mutations are responsible for MEB (Yoshida et al., 2001; Taniguchi *et al.,* 2003; Vervoort *et al.,* 2004). Mutations in the FKRP gene have also been identified in MEB patients (Beltran-Valero de Bernabe *et al.,* 2004).

Muscular Dystrophy, Duchenne Type (DMD; MIM 310200)

DMD is described as muscle weakness and respiratory and cardiac malfunction. Mutations in Dystrophin lead to DMD (Zneimer *et al.*, 1993). DMD gene was found to be linked on chromosomal region Xp21 (Lindenbaum *et al.,* 1979). Kingston *et al.,* (1983, 1984) and Francke *et al.,* (1985) confirmed these findings.

Emery-Dreifuss Muscular Dystrophy (EDMD)

EDMD is characterized as joint deformity, skeletal muscle changes snf hesrt problem. Two Syndromes of Emery-Dreifuss have been described yet. EDMD2 (MIM 181350), caused by mutation in Laminin A/C gene is inherited in autosomal dominant mode while EDMD3 (MIM 604929) shows autosomal recessive mode of inheritance.

EDMD, X-Linked (edmd; MIM 310300 is inherited as an X-linked recessive disorder, although autosomal dominant mode of inheritance has also been reported

(Rudenskaya *et al.,* 1994). Boswinkel *et al.,* (1985) reported linkage for EDMD on chromosomal region Xq28. This region was further refined by Thomas *et al.,* (1986), Romeo *et al.,* (1988), Consalez *et al.,* (1991) and Yates *et al.,* (1993). Mutation causing EDMD has been recognized in Emerin (EMD; MIM 300384) coding gene (Hoeltzenbein et al., 1999; Ben Yaou et al., 2007).

By genetic linkage analysis of a large affected French pedigree, Bonne *et al.,* (1999) mapped the locus for EDMD2 on chromosome 1q11-q23. Mutations causing EDMD2 in the LMNA gene have been identified (Bonne *et al.,* 1999; Muchir *et al.,* 2000). In a 40-year-old man with severe EDMD, Raffaele di Barletta *et al.,* (2000) identified heterozygous mutations causing EDMD3 in the LMNA gene located on chromosome 1q21.2.

Facioscapulohumeral Muscular Dystrophy

Facioscapulohumeral muscular dystrophy (FSHMD) is a highly variable disorder with weakness appearing from childhood to late life but normally in the second decade. In general, the disease initially involves the face and the scapulae followed by the foot dorsiflexors and the hip girdles. Mathews *et al.,* (1991) defined the location of FSHD Gene 1 (FRG1; MIM 601278) as 4q35. Gilbert *et al.,* (1993) found evidence for heterogeneity in FRG1.

Oculopharyngeal Muscular Dystrophy (OPMD; MIM 164300)

It is an autosomal dominant disorder and is characterized by dysphagia and progressive drooping of eyelids (Victor *et al.,* 1962). By linkage analysis of a homogeneous group of OPMD families, Brais *et al.,* (1995) identified a putative disease locus within a 5-cM segment of chromosome 14q11.2-q13. Teh *et al.,* (1997) reported Australian kindred of German descent with OPMD. Linkage analysis supported the previous assignment to 14q. Brais *et al.,* (1995) suggested that the Myosin, Heavy Chain 7, Cardiac Muscle, Beta gene (MYH7; MIM 160760) or the contiguously situated Myosin, Heavy Chain 6, Cardiac Muscle, Alpha gene (MYH6; MIM 160710) could be the cause of this disorder.

Dystrophia Myotonica

Myotonic dystrophy (DM) is characterized by Muscle wekness, Frontal balding and myotonia. It shows autosoam dominant mode of inheritance (Harper, 1975; Groh *et al.,* 2008). The classic form of the disorder, myotonic dystrophy-1 (DM1; MIM

160900), is caused by CTG repeat in the dystrophia myotonica protein kinase gene (DMPK; MIM 605377) on 19q13 (Eiberg *et al.,* 1983). Dystrophia Myotonica 2 (DM2; MIM 602668) is caused by expansion of a CCTG repeat in intron 1 of the zinc finger protein-9 gene (ZNF9; MIM 116955) on 3q13.3-q24 (Ranum *et al.,* 1998).

Distal Muscular Dystrophy

This group of disorders is characterized by weakness and wasting of small muscles of hands, slowly progressive and apparently did not shorten life (Welander, 1957). Autosomal Recessive mode of inheritance for Myopathy, Distal, With Anterior Tibial Onset (DMAT; MIM 606768) has also been reported (Illa *et al.,* 2001). Dysferlin gene (DYSF; MIM 603009) is considered as a key candidate gene for all forms of Distal Muscular Dystrophy i.e DMAT, Welander Distal Myopathy (WDM; MIM 604454) and Miyoshi Myopathy (MM; MIM 254130) (Ahlberg *et al.,* 1999) and all forms are localized on chromosome 2p13.3-p13.1 (von Tell *et al.*, 2003).

Limb-Girdle Muscular Dystrophy

Limb-Girdle Muscular Dystrophy (LGMD) characterized by proximal muscle weakness, starts in the hip girdle region and later affects the shoulder girdle region. It shows autosomal dominant mode of inheritance (Schneiderman *et al.,* 1969).

Several forms of autosomal dominant LGMD has been identified which include Muscular Dystrophy, Limb-Girdle, Type 1A (LGMD1A; MIM 159000), localized on 5q31 (Speer *et al.,* 1992), with autosomal dominant inheritance, caused by mutation in the gene encoding myotilin (TTID; 604103) (Hauser *et al.,* 2000, 2002); Muscular Dystrophy, Limb-Girdle, Type 1B (LGMD1B; MIM 159001), mapped on 1q21.2 (Van der Kooi *et al.,* 1997), shows autosomal dominant inheritance (Van der Kooi *et al.,* 1996, 1997), caused by mutation in LMNA (Muchir *et al.,* 2000; van Engelen *et al.,* 2005); Muscular Dystrophy, Limb-Girdle, Type 1C (LGMD1C; MIM 607801), caused by mutation in the caveolin-3 gene (CAV3; MIM 601253) which is located on 3p25 and shows autosomal dominant inheritance (Minetti *et al.,* 1998; McNally *et al.,* 1998; Betz *et al.,* 2001; de Paula *et al.,* 2001); Muscular Dystrophy, Limb-Girdle, Type 1D (LGMD1D; MIM 603511), Localized on 7q (Speer *et al.,* 1999), shows autosomal dominant inheritance (Chutkow *et al.,* 1986); Muscular Dystrophy, Limb-Girdle, Type 1E (LGMD1E; MIM 602067), localized on 6q23 (Messina *et al.,* 1997); Muscular Dystrophy, Limb-Girdle, Type 1F (LGMD1F; MIM 608423), localized on

7q32.1-q32.2 (Gamez *et al.,* 2001, Palenzuela *et al.,* 2003); Limb-Girdle Muscular Dystrophy, Type 1G (LGMD1G; 609115) localized on chromosome 4q21 (Starling *et al.,* 2004); Muscular Dystrophy, Limb-Girdle, Type 2A (LGMD2A; MIM 253600), localized on 15q15.1-q21.1, with autosomal recessive inheritance and is caused by mutation in the gene encoding the proteolytic enzyme calpain-3 (CAPN3; MIM 114240) (Passos-Bueno *et al.,* 1993; Fougerousse *et al.,* 1994; Allamand *et al.,* 1995); Muscular Dystrophy, Limb-Girdle, Type 2B (LGMD2B; MIM 253601), localized on 2p13.3-p13.1 (Bashir *et al.,* 1996), with autosomal recessive inheritance (Bashir *et al.,* 1994), caused by mutations in the gene encoding DYSF (Bashir *et al.,* (1998); Muscular Dystrophy, Limb-Girdle, Type 2C (LGMD2C; MIM 253700) localized on 13q12 (Ben Othmane *et al.,* 1992) with autosomal recessive inheritance (Hazama *et al.,* 1979) and caused by mutation in the gamma-sarcoglycan gene (SGCG; MIM 608896) (Noguchi *et al.,* (1995); Muscular Dystrophy, Limb-Girdle, Type 2D (LGMD2D; MIM 608099), localized on 17q12-q21.33 with autosomal recessive inheritance and caused by alpha-sarcoglycan gene (SGCA; MIM 600119) mutation (Roberds *et al.,* 1994; Passos Bueno *et al.,* 1995; Piccolo *et al.* 1995; Trabelsi *et al.* 2008); Muscular Dystrophy, Limb-Girdle, Type 2E (LGMD2E; MIM 604286), localized on chromosome 4q12, shows autosomal recessive inheritance and is caused by beta-sarcoglycan (SGCB; MIM 600900) mutations (Lim *et al.,* 1995; Bonnemann *et al.,* 1996; Trabelsi *et al.,* 2008); Muscular Dystrophy, Limb-Girdle, Type 2F (LGMD2F; MIM 601287) localized on 5q33-q34, shows autosomal recessive inheritance and caused by sarcoglycan-delta gene (SGCD; MIM 601411) mutations (Nigro *et al.,* 1996; Passos-Bueno *et al.,* 1996; Moreira *et al.,* 1998; Trabelsi *et al.,* 2008); Muscular Dystrophy, Limb-Girdle, Type 2G (LGMD2G; MIM 601954) linked to region on chromosome 17q11-q12 and is caused by mutation in the gene encoding telethonin (TCAP; MIM 604488) (Moreira *et al.,* 1997, 2000); Muscular Dystrophy, Limb-Girdle, Type 2H (LGMD2H; MIM 254110) shows autosomal recessive inheritance and is due to mutation in the gene encoding tripartite motif-containing protein-32 (TRIM32; MIM 602290) on chromosome 9q31-q34.1 (Weiler at al., 1998; Frosk *et al.,* 2002) (Weiler *et al.,* 1998; Frosk *et al.,* 2002); Muscular Dystrophy, Limb-Girdle, Type 2I (LGMD2I; MIM 607155) localized on 19q13.3, shows autosomal recessive inheritance, caused by FKRP gene mutations (Driss *et al.,* 2000, Brockington *et al.*, 2001; de Paula *et al.*, 2003; Harel *et al.*, 2004; Frosk *et al.*,

2005); Muscular Dystrophy, Limb-Girdle, Type 2J (LGMD2J; MIM 608807) localized on 2q31 and caused by titin (TTN; 188840) gene mutations (Udd *et al.*, 1992, Haravuori *et al.*, 1997, 1998, 2002); Muscular Dystrophy, Limb-Girdle, Type 2K (LGMD2K; MIM 609308) caused by mutation in POMT1on 9q34 (Dincer *et al.*, 2003, Balci *et al.*, 2005; D'Amico *et al.*, 2006); Muscular Dystrophy, Limb-Girdle, Type 2L (LGMD2L; MIM 611307) localized on 11p13-p12 (Jarry *et al.*, 2007).

ANALYSIS USING BIOINFORMATICS APPROACH

Candidate Disease Gene Identification

Disease Gene Identification by employing Genome wide search methods e.g. linkage analysis produce large sets of candidates and also it remains complicated to spot the disease related genes. Bioinformatics has breached this barrier by introducing computational approaches to identify candidate diseases genes. Computational methods developed so far extract information regarding Candidate disease genes by searching through different biological, functional and structural data sources (Kanehisa and Bork, 2003; Stein, 2003).

Existing methods for candidate gene identification can be classified into two categories i.e. Methods based in Functional Annotation and Methods based on Sequence similarity. Tiffin *et al.*, (2006) reviewed seven disease gene identification methods and also identified most likely disease genes for type 2 diabetes (T2D) and the related trait obesity. Different tools are being used for candidate gene identification such as GeneSeeker (van Driel *et al.*, 2005); G2D (Genes2Diseases) (Perez-Iratxeta *et al.*, 2005); PROSPECTR and SUSPECTS (Adie *et al.*, 2006); GeneWanderer (Kohler *et al.*, 2008); GeneDistiller (Seelow *et al.*, 2008); PosMed (Yoshida *et al.*, 2009). Zhu and Zhao (2007) reviewed the main research advances in the subject of candidate gene approach, the strategies for breaking the information holdup by traditional candidate gene approach and also discussed a new candidate gene finding approach i.e. digital candidate gene approach (DigiCGA).

Docking Analysis of Different Compounds for Treating Muscular Dystrophy

Structure based or rational drug discovery has resulted in variety of drugs in the market and many more are under development. Structure based methods are used in almost all steps of drug development. Three dimentional (3D) structure of receptor or

Ligand-receptor comple is main point of focus in all these methods because the structure of the target determines which ligands it binds. Developments of computational methods to determine protein structures have enhanced the applicability, accuracy, and speed of structural studies (Eswar and Sali, 2007).

Recent research, regarding treatment of different types of Muscular Dystrophies, is based on finding non-drug approaches. In this regards, most of the attention has been focused on Antioxidants as this diverse group of bioactive compounds play important role in health maintainance and disease prevention by protecting other chemicals and substances from oxidation by being oxidized itself.

Oxidative stress and Free radical damage is involved in the succession of Muscular Dystrophies studied to date (Toscano *et al.,* 2005). Ihara *et al.,* (1995) and Tidball *et al.,* (2007) described in their study that oxidative stress and free radical overload was found to be involved both in human and mouse suffering with muscular Dystrophy. In another study, food supplementd with different antioxidants was given to dystrophic mice for several weeks and significant improvement in muscle power was observed. Such results leads to the recommendation of antioxidants as possible therapy fo Muscular Dystrophy (Buetler *et al.,* 2002; Dorchies *et al.,* 2006; Messina *et al.,* 2006). The Duchenne and Becker Muscular Dystrophies are caused by mutation in Dystrophin protein, a large muscle protein, encoded by DMD gene. For the present study Asparagine-231- tyrosine substitution, reported by Roberts *et al.,* (1994) was selected for docking analysis.

Following compounds have been selected for computational analysis, as possible cure for muscular dystrophy:

1. Triterpenes Antioxidants i.e. Ganoderic acids A, B, C, and D; Ganoderol A, B and Ganodermanontriol found in Reishi Mushroom.
2. Supplements i.e. Creatine and Coenzyme Q10

The Reishi Mushroom, purplish-brown fungus, is being used in Chinese medicine for more than 4,000 years for treating fatigue, asthma, cough, liver ailment and to support long life. It is native to China, Japan, and North America but is cultivated throughout other Asian countries (Lininger *et al.,* 1998; Li *et al.,* 2007). It has been evaluated that Reishi has curing affect for Cancer, Hepatitis, retinal pigmentary degradation, ulcers, insomnia and Muscular Dystrophy (Chang and But, 1986).

In Reishi Mushroom at least 36 different compounds have been identified (Paterson, 2007), Triterpene constituents have also been analyzed (Kohda *et al.,* 1985). Triterpene antioxidants, including ganoderic acids A, B, C, and D; Ganoderol A and B; Lucidenic acid B, and ganodermanontriol (Figure 1.1) have been identified (Lininger *et al.,* 1998; Zhu *et al.,* 1999; Hajjaj *et al.,* 2005; Tang *et al.,* 2006; Liu *et al.,* 2007; Paterson, 2007).

Creatine (Figure 1.2) is a nitrogenous organic acid found mainly in muscles and help in supplying energy to nerve and muscle cells. Half of the creatine is synthesized in Kidney, pancrease and Liver while rest is taken through foods. It is present in the form of Creatine Phosphate or phosphocreatine. Catalysis of Creatine produces Creatine and phosphate while energy which is released during this process is used for ATP regeneration. Creatine is continuously broken down and converted to its metabolite by-product creatinine, which diffuses out of the muscle cells and is excreted by the kidneys into the urine (Wyss and Kaddurah, 2000).

Creatine supplements are being studied continuously as a possible therapeutic approach for the treatment of muscular, neuromuscular, neurological and neurodegenerative disorders. Studies have shown that Creatine supplements with exercise are effective in improving muscle function (Chung *et al.,* 2007).

Coenzyme Q10 (CoQ10) (Figure 1.3), a vitamin-like compound, is present in the mitochondria of all body cells. It is involved in ATP synthesis by providing help in transfer of electrons in respiratory chain.CoQ10 is active as an antioxidant and protects the cell from oxidative damage. The level of CoQ is maintained by Dietry sources and body's own production. It is widely used as supplement for treating Oxidative Phosphorylation (OXPHOS) disorders and it has been found effective against mitochondrial disorders. major positive effects include improved neurological function (Bresolin *et al.,* 1988), reduced muscle weakness (Yamamoto *et al.,* 1987; Ihara *et al.,* 1989; Abe *et al.,* 1991) and decrease in nerve damage (Ihara *et al.,* 1989).

Effectiveness of CoQ10 was checked and improvement in ATP synthesis and phosphorylation in both skeletal and brain muscles was observed (Chen *et al.,* 1997; Barbiroli *et al.,* 1997). Significant improvement in muscle weakness was observed after administration of CoQ10 in patients with CoQ10 deficient (Musumeci *et al.,* 2001).

In present study, Pakistani families with Limb Anomalies i.e. Syndactyly, Polydactyly and with Muscular Dystrophy have been ascertained. The data presented here includes Linkage studies carried out for all ascertained families, candidate disease gene identification for PAP-A3 by web based tools and computational analysis of available supplements and various Triterpene antioxidants as possible therapies for different types of Muscular Dystrophy by using docking Tools.

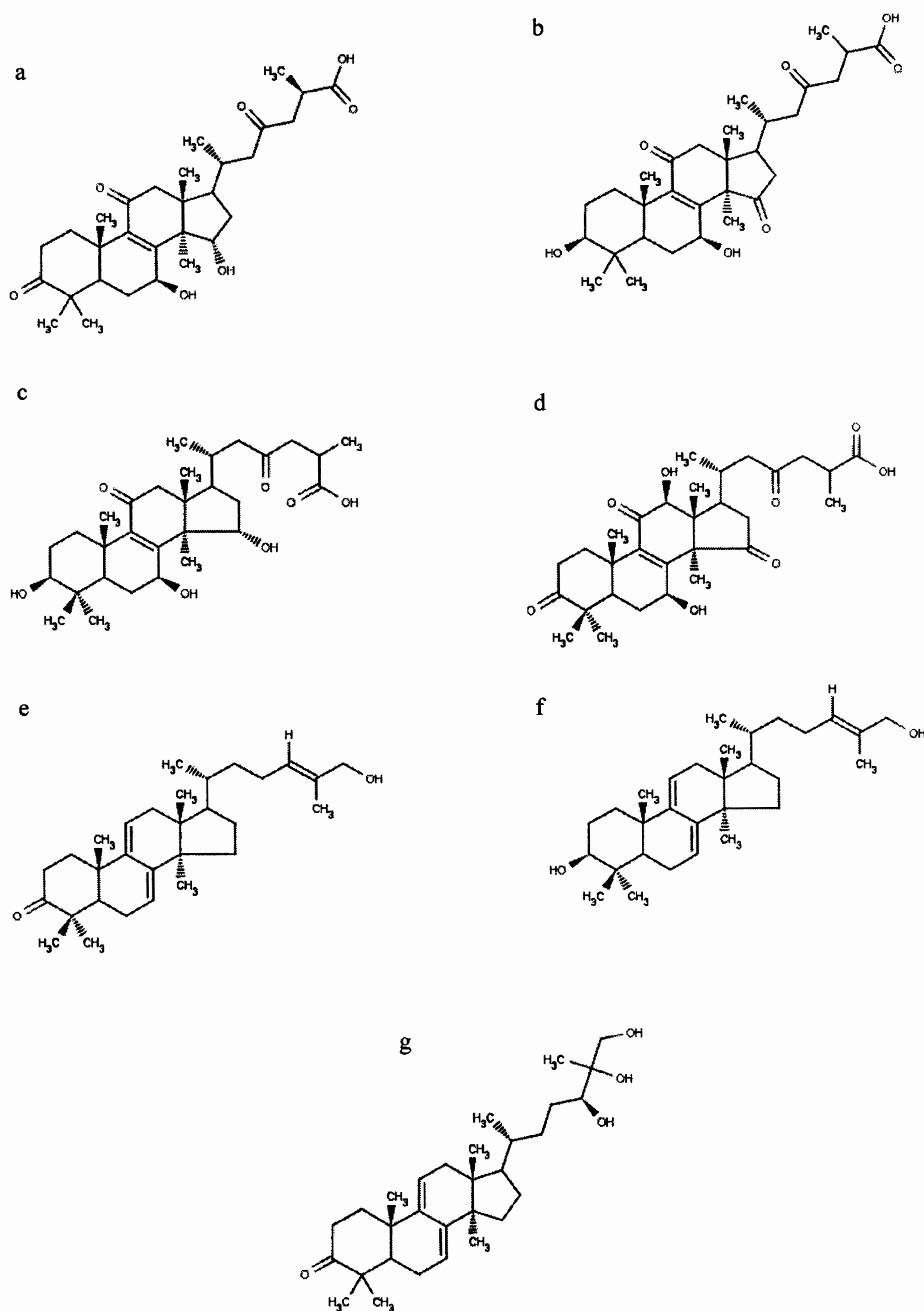

Figure 1.1: Triterpenes Antioxidants i.e. Ganoderic acids A (a), B (b), C (c), and D (d); Ganoderol A (e) and B (f) and Ganodermanontriol (g).

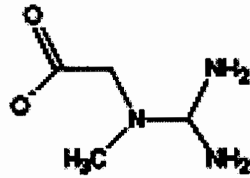

Figure 1.2: Structure of Creatine.

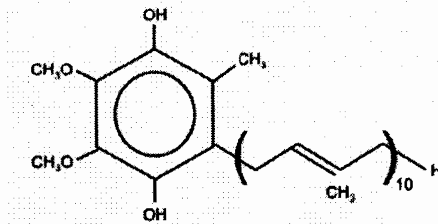

Figure 1.3: Structure of Coenzyme Q10.

MATERIALS AND METHODS

This study was approved by Instititional Board o Study, International Islamic University, Islamabad. Acquiescence was taken from the parents of the affected children and all other members of the family who took part in the study. Written consent was also taken from the parents of the affected children to publish their photographs.

Families with Limb Anomalies

Two Pakistani families referred hereafter as A and B, with Limb anomalies i.e. Syndactyly and Polydactyly respectively, were studied from NWFP and Punjab regions of Pakistan. Information regarding family history of each family member was collected.

Families with Muscular Dystrophies

Two Pakistani families referred hereafter as C and D, showing muscular Dystrophies, were studied from Punjab regions of Pakistan. Information regarding family history of each family member was collected.

Pedigree Analysis

For genetic implication an extensive pedigree was constructed for each family by the standard methods described by Bennett *et al.,* (1995). The pattern of inheritance of hereditary limb anomalies and Muscular Dystrophies was worked out by observing the segregation of these disorders within family. The exact genealogic relationships for all the affected individuals were obtained through the extensive personal interviews of elders of the families. Circles and squares were used to symbolize females and males, respectively. The normal and affected individuals were designated with unfilled and filled symbols respectively. Roman numeral was used to indicate each generation.. The individual within a generation were designated by Arabic numerals.

Blood sampling

Blood samples were drawn by 5 c.c. syringes and vacutainer tubes containing Acid Citrate Dextrose (ACD). The blood was stored at 4°C until DNA extraction.

Genomic DNA extraction

Phenol Choloroform method was employed to extract and purify genomic DNA from peripheral blood lymphocytes.

1. Five to 8 ml blood was collected in 50 ml falcon tube.
2. To the blood sample, three times of Cell lysis Buffer was added and was stored on ice for 30 minutes.
3. After chilling, Separation of white blood cells was carried out by centrifugation at 1200 rpm for 10 minutes at 4°C .
4. The supernatant was discarded and the pellet was resuspended.
5. Firstly 4.75 ml of STE was added and then 250 µl of 10% SDS was added drop-wise while vortex.
6. 10 µl of protinase-K (20 mg/ml) was added and was incubated overnight at 55°C.
7. On the following day, 5ml of mixture of equilibrated phenol and chilled chloroform-Isoamylalcohol (1:1) was added and tube was shaken for 10 minutes and then was kept on ice for 10 minutes.
8. After chilling, centrifugation was carried out at 3200 rpm for 30 minutes at 4°C and aqueous layer was removed with cut tip and was transferred into separate labeled 15ml falcon tube.
9. 10 µl of RNase (10 mg/ml) was added to this aqueous layer and was incubated in water bath shaker at 37°C for 2 hours.
10. 250 µl of 10% SDS was added drop wise without vortex and then 10 µl of protinase-K (20 mg/ml) was added was incubated at 55°C for 1 hour.
11. Step 7 and 8 was repeated to obtain a clean supernatant containing genomic DNA.
12. The clear supernatant was transferred to a new 15ml falcon tube and the DNA was precipitated by the addition of 500 µl of 10M Ammonium Acetate and 5 ml of Isopropanol.
13. The tube was placed at -20°C overnight.
14. Next day, centrifugation was carried out at 3200 rpm for 60 minutes, supernatant was discarded and pallet was resuspended.
15. The pallet was washed with 5 ml of chilled 70% ethanol at 3200 rpm for 40 minutes at 4°C. Supernatant was discarded and pallet was dried.

16. The pallet was re-suspended in 10 mM Tris-HCl (PH 8.0).
17. Genomic DNA was quantified by taking Optical Density (OD) of sample at 260 nm and 280 nm by Spectrophotometer (Bio-Rad Laboratories, USA).
18. The dilution of 100 ng/μl of each stock sample was prepared for PCR amplification.

Genotyping

To carry out the linkage or exclusion studies of the families to known loci (five for Syndactyly and four for polydactyly), a minimum of two microsatellite markers were used for each of the candidate regions of these loci in all the available individuals of the four families (A, B, C and D). List of microsatellite markers which were utilized for genotyping these families are shown in Table 2.1 and 2.2.

Forty Seven loci for nine different types of Muscular Dystrophies have been identified to date. Two families of muscular dystrophies (C and D) were first tested for linkage to twenty two known loci. Microsatellite markers used for genotyping these families are shown in Table 2.3.

Microsatellite markers mapped by Cooperative Human Linkage Center (CHLC) were obtained from Research Genetics, Inc. (USA). The cytogenetic locations of these markers, their heterozygosity as well as the sizes of the amplified products were obtained from LDB Genetic Map (H.H. Stassen, Psychiatric University Hospital Zurich, Switzerland).

Polymerase chain reaction (PCR)

PCR was carried out in a total volume of 10μl, having 40 ng/μl of genomic DNA from DNA dilutions, 0.25 μl of each primer (20 μM stock), 1 μl 10X PCR buffer with $MgCl_2$, (100 mM of Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 1 μl of dNTPs (2.5mM stock), 5.6 μl of dH_2O and 0.4 μl of Taq DNA polymerase (3U/μl) (BIOMATIK). The master mix was centrifuged for 10 seconds for thorough mixing. PCR was carried out in Gene Amp PCR system 9700 (Applied Biosystems, USA).

Horizontal gel electrophoresis

The amplification of the genomic region was checked on 2% agarose gel, which was prepared by melting 6 g. of agarose in 350 ml 0.5X TAE buffer (0.89 M Tris, 0.025 M Borate, EDTA PH 8.3) in a microwave oven for few minutes. Ethidium bromide (final concentration 0.5 μg/ml) was added to the gel to facilitate visualization of DNA after electrophoresis. PCR reaction products were mixed with Bromophenol blue dye (0.25% Bromophenol Blue in 40% sucrose solution) and loaded into the wells. Electrophoresis was performed at 100 Volts for half an hour in 1X TBE buffer. Amplified products were detected by placing the gel on UV transilluminator (Biometria, Germany).

Polyacrylamide Gel Electrophoresis

1. Master Mix was prepared as given below by adding reagents in sequential order.

Master Mix	**Final Conc.**	**Required Volume per reaction μl**
Deionizad H_2O		5.6
10X PCR Buffer	1 X	1
2.5 Mm dNTPs	100 μM	1
Taq Polymerase(2.5 U/μL)	2.5 U	0.4
20 μM Forward Primer	600 nm	0.25
20 μM Reverse Primer	600 nm	0.25
Total		8.5

2. 40ng/μl DNA and 8 μl master mix was added to each tube.
3. DNA was amplified under the above mentioned PCR thermal cycler conditions:

Step	Temperature	Duration	Cycles
Denaturation	95°C	5 min	1
Denaturation	95°C	45 sec	
Annealing	55°C	1 min	35
Extension	72°C	1 min	
Final Extension	72°C	10 min	1

4. 10% Polyacrylamide gel solution was prepared by adding 50 ml of 40% Acrylamide solution and 25 ml of 10X TBE q.s. to 250 ml with deionized water.
5. 300 µl of 25 % APS (Ammonium Per Sulphate) and 300 µl of TEMED was added in 50 ml from the 250 ml of 10 % Acrylamide solution and was poured into the base of Biorad SeqiGen System and allowed to polymerize for 2-3 minutes.
6. To the remaining 200 ml of 10% Acrylamide solution, 850 µl 25% APS solution and 150 µl of TEMED was added and Poured and allowed to polymerize for at least 2 hours.
7. 1X TBE Buffer was added in the upper and lower buffer chambers of the gel unit. After Setting up the sequencing system, it was pre-run for 10-15 minutes at 100 watts constant power.
8. After Taking out the comb, wells were washed with 1X TBE Buffer.
9. 5 µl gel loading dye was mixed with amplified product. DNA molecular weight marker, in the first lane of the gel, and then 8 µl of the amplified products were loaded in the appropriate wells. (1.4 µl of PCR products was mixed with 5µl gel loading dye containing Bromophenol Blue and Xylene Cyanol, and analysed on 10% denaturing polyacrylamide gel.
10. Electrophoresis was performed at 100 watts for 4-5 hours. After cutting the gel according to expected band sizes, it was stained with Ethidium Bromide for a few minutes.
11. Amplified products were detected by placing the gel on UV transilluminator and gel photographs were analyzed to determine alleles.
12. SynGen was used to perform fragment analysis and software used was GeneScan (version 3.1.2).

Reagents used in gel solution

1. **10X Tris Boric Acid EDTA buffer** (TBE; pH 8.2)
    1. 108 gm Tris, 55 gm Boric Acid and 7.44 gm EDTA were dissolved up to 0.8 L of deionized water on hot stirrer.
    2. Volume of solution was raised up to 1 L in a cylinder.
    3. pH of solution was adjusted at 8.03.
    4. It was filtered and transferred to labeled bottles and stored at room temperature.
2. **Ammonium Per Sulphate (APS) 25% (w/v)**

    25 grams of APS was dissolved in 100 ml of distilled water labeled and stored in the refrigerator.

Linkage analysis

Cyrillic 2.1.3 was used to manage and record pedigree and genotype data for linkage analysis. MLINK i.e. a program of LINKAGE software package (version 5.1), was used to calculate the Two-point Lod Score (Lathrop *et al.,* 1984). Analyses were automated by using linkage support programs (LSP, LCP, MAKEPED, PREPLINK). For Family B, an autosomal dominant mode with 0.999 penetrance and 0.001 disease allele frequency was assumed. For fine mapping, the allele frequencies of markers were either considered from the founders of family or assumed as equal. Analyses were also conducted by using the online facility of GLUE (Genetic Linkage User Environment; www.rfcgr.mrc.ac.uk/).

Candidate Disease Gene Identification

To select the disease related gene, results of association studies in postaxial polydactyl family were used as a prime filter. Genomic locations and genes known in mapped loci were gathered from ENSEMBL (www.ensembl.org) as our starting candidate genes. Following tools were used to generate the list of candidate genes:

G2D

Candidate genes are predicted by G2D (Perez-Iratxeta *et al.,* 2005) by scoring sequence fragments of chromosomal region by comparing them against standard database of genes. Depending upon GO Score and R-Score, hundred disease candidate genes, responsible for Postaxial Polydactyly, were estimated by G2D. List

was further refined and selection of five best candidates was made by the functional analysis of genes and their involvement in genetic disorders.

PROSPECTR and SUSPECT

Suspect was used to score genes present in mapped locus. The genomic area to be searched for candidate gene was limited by marker position. Nearby genes to the marker were selected and then scored by PROSPECTR (Adie *et al.,* 2006).

GeneWanderer

GeneWanderer is a web-based computational program which pritorizes a gene by calculating probability of its involvment in particular phenotype or disease (Kohler *et al.,* 2008).

GeneDistiller

GeneDistiller (Seelow *et al.,* 2008) is a web-based application which integrates information from various data sources such as gene-phenotype associations, gene expression patterns and protein-protein interactions into a central database.

PosMed (Positional Medline)

PosMed priortizes a gene by its biological interactions with other genes, metabolite, drug, disease and also considers its protein-protein interactions (Yoshida *et al.,* 2009).

Docking Studies

A detailed literature survey was carried out to find out the ligands for mutated Dystrophin protein. The structure of Triterpene Antioxidant in mol file format was retrieved from chemspider (http://www.chemspider.com/) which were converted in pdb file format by using Marvinbeans-5.2.2 (http://www.chemaxon.com/download.html). Target protein i.e. Dystrophin (1DXX.pdb) was retrieved from Protein Data Bank (Bernstein *et al.*, 1977). WHAT-IF server (Vriend, 1990) was used for inserting above described mutation. Mutated Dystrophin was docked with selected ligands by using Autodock tools version 3.0.5 (Morris *et al.,* 1998) which makes computational predictions of molecular interactions and calculate the docking energies of ligand-receptor Complex, and Hex 5.0 developed by Ritchie and Graham (2000) which carried out Macromolecular Docking by using Spherical Polar Fourier correlations.

Table 2.1: Microsatellite markers used to test linkage to known Syndactyly Loci

Locus	Cytogenetic Location	Markers	Distance(cM) Rutgers Map Build 36	Amplified Length (bp)	Variation Type
SYNDACTYLY, TYPE I	2q34-q36	D2S1776	168.1	298	TTR
		D2S1391	193.2	121	TTR
	3p21.31	D3S2409	60.6	118	TNR
SYNDACTYLY, TYPE II	2q31	D2S1776	168.1	298	TTR
		D2S1391	193.2	121	TTR
	22q13.3	D22S445	45.22	120	TNR
SYNDACTYLY, TYPE III	6q22-q24	D6S1040	128.2	271	TTR
		D6S474	123.8	155	TTR
SYNDACTYLY, TYPE IV	7q36	D7S1823	173.71	221	TNR
		D7S559	169.1	209	DNR
SYNDACTYLY, TYPE V	2q31–q32	D2S1776	168.1	298	TTR
		D2S1391	193.2	121	TTR

Table 2.2: Microsatellite markers used to test linkage to known Polydactyly Loci

Phenotype	**Locus**	**Markers**	**Distance(cM) Rutgers Map Build 36**	**Amplified Length (bp)**	**Variation Type**
PAP-A1	7p13	D7S3046 D7S1818	67.1 62.0	332 191	TTR TTR
PAP-A2	13q21-q32	D13S317 D13S793	85.1 95.8	188 263	TTR TTR
PAP-A3 (PAP-A/B)	19p13.2-p13.1	D19S586 D19S433	11.3 37.8	236 1207	TTR TTR
PAP-A4	7q22	D7S1799	115.6	188	TTR

Table 2.3: Microsatellite markers used to test linkage to known Muscular Dystrophies Loci

Locus	Cytogenetic Location	Marker	Distance (cM) Rutgers Map Build 36	Amplified Length (bp)	Variation Type
MDC1A	6q22-q23	D6S1040	128.2	271	TTR
		D6S474	123.8	155	TTR
UCMD	2q37 21q22.3 21q22.3	D21S1446	49.7	217	TTR
		D21S2055	46.3	155	TTR
		D2S2968	253.1	183	TTR
		D2S427	247.9	253	TTR
WWS	14q24.3 9q34.1 9q31 22q12.3-q13.1 19q13.3	D9S158	139.5	223	DNR
		D9S2157	146.54	267	TNR
		D14S553	91.5	271	TTR
		D14S606	85.9	270	TTR
MEB	1p34-p33 19q13.3	D1S1586	56.1	105	TNR
		D1S2134	59.5	275	TTR
		D19S178	48.5	167	DNR
		D19S246	57.8	207	DNR
FCMD	9q31	D9S934	123.7	218	TTR
		D9S930	119.8	294	TTR
		D9S938	106.3	413	TTR
MDC1B	1q42	D1S3462	246.2	259	TNR
		D1S549	239.5	179	TTR
MDC1C	19q13.3	D19S178	48.5	167	DNR

		D19S246	57.8	207	DNR
MDC1D	22q12.3-q13.1	D22S685 D22S689	42.2 40.5	190 214	TTR TTR
RSMD1S	1p36-p35	D1S1622 D1S552	32.5 22.6	261 256	TNR TTR
BMD	Xp21.2	GATA144D04 DXS6810	45.1 42.7	238 215	TTR TTR
DMD	12q21 Xp21.2	GATA144D04 DXS6810	45.1 42.7	238 215	TTR TTR
MM	2p13.3-p13.1	D2S1394 D2S2739	72.7 62.6	168 305	TTR TTR
EDMD	Xq28	DXYS154 DXS9908	159.9 143.9	239 231	DNR TTR
FSHMD1A	4q35	D4S1652	203.0	140	TTR

CONGENITAL LIMB ANOMALIES

A wide variety of congenital limb anomalies reflect the complexity and precision of limb development. Identification and characterization of the underlying gene(s) can increase our understanding of normal limb development. I got the possibility to study two Pakistani families with Syndactyly and postaxial polydactyly.

SYNDACTYLY

Non-syndromic Syndactyly is a common, heterogeneous hereditary condition of webbed fingers and/or toes. It is two times more common in males. The distribution of webbing between the digits varies (Adrian *at al.*, 2005). Syndactyly has a frequency of 1:2000 in the population. It can be unilateral or bilateral, and the fusion within the web may be cutaneous or bony (de Smet *et al.*, 1994).

On the basis of an anatomic approach, Non-Syndromic Syndactylies has been subdivided into eight major types (Temtamy and McKusick, 1978; Goldstein *et al.*, 1994). Syndactyly type I is described by complete or partial Webbing of 3rd and 4th fingers and/or 2nd or 3rd toes (Castilla *et al.*, 1980). Syndactyly type II is characterized as a cutaneous or bony fusion between the middle and ring fingers associated with complete or partial duplication of the ring finger in the web (Temtamy and McKusick, 1978). Syndactyly type III is a complete and bilateral Syndactyly between the fourth and fifth fingers (Paznekas *et al.*, 2003). Type III Syndactyly has been reported as a part of Oculo-dento-digital Dysplasia (ODD; MIM 16420). Syndactyly Type IV is characterized by complete fusion of all fingers in both hands (Haas *et al.*, 1940). Syndactyly Type V is a postaxial Syndactyly. Syndactyly Type VI is characterized by a webbing of digits 2-5 in both hands and feet (Temtamy and McKusick, 1978). Syndactyly Type VII is characterized by complete Syndactyly of hands and feet, abnormal phalanges, carpal and metacarpal fusion, giving the hand a spoon-like appearance (Cenani and Lenz, 1967). Syndactyly type VIII shows unilateral or bilateral fusion of metacarpal 4th and 5th (Lonardo *et al.*, 2004).

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All types of Syndactylies show autosomal dominant mode of inheritance, with variable expression and incomplete penetrance while Syndactyly type VI is the only type which is autosomal recessively inherited (Cenani and Lenz, 1967).

Syndactyly types I, II, III, IV and V have been mapped to chromosomal regions 2q34–q36, 2q31–q32, 6q21–q23.2, 7q36 and 2q31–q32 respectively (Sarfarazi *et al.*, 1995; Gladwin *et al.*, 1997; Bosse *et al.*, 2000; Sato *et al.*, 2007) while no linkage has been reported yet for Syndactyly types VI, VII, VIII (Cenani and Lenz, 1967; Temtamy and McKusick, 1978; Lonardo *et al.*, 2004). Malik *et al.* (2005) predicted genetic heterogeneity in zygodactyly. He localized a new gene for type 1 Syndactyly on 3p21.31 by genomewide linkage analysis in a Pakistani family and proposed to designate the 3p21.31 locus as ZD1. Malik *et al.* (2006) reported genetic heterogeneity for SDP and localized a new gene for Synpolydactyly at chromosome 14qll.2-ql2.

Many different genes appear to be involved in limb development. WNT6 is a promising candidate gene for Syndactyly type I (Parr *et al.*, 1993). Synpolydactyly is described to be cause by mutations in FBLN1 (Debeer *et al.*, 2002) and HOXD13 (Kan *et al.*, 2003). GJA1 has significant role in causing Syndactyly type III (Vitiello *et al.*, 2005). Mutations in LMBR1 gene are described to be involved in thumb-polysyndactyly syndrome (Wang *et al.*, 2007). Syndactyly type IV is reported to be caused by mutations in SHH (Sun *et al.*, 2008) and HOXD13 (Zhao *et al.*, 2007).

POSTAXIAL POLYDACTYLY

Postaxial Polydactyly (PAP) is described by the presence of one or more extra finger and/or toe on the ulnar or fibular side of the hands and feet, respectively. Phenotypically it is subdivided into two types. In Postaxial PAP-A, the extra digit is rather well developed, articulates with the fifth, or extra, metacarpal/metatarsal, and is usually functional while PAP-B the extra digit is not well formed and mostly it is present in the form of a skin tag (Temtamy and McKusick 1969).

Isolated PAP is usually inherited as an autosomal dominant trait, with variable penetranve and expressin while autosomal recessive trait has also been reported (Mohan 1969; Cantu *et al.,* 1974; Mollica *et al.,* 1978). Currently, there are four loci for Postaxial Polydactyly in humans. PAP-A1 on Chromosome 7p15-q11.23, PAP-A2 on chromosome 13q21-q32, PAP-A3 on chromosome 19p13.1-13.2 and PAP-A4 on chromosome 7q21–q34 has been mapped (Akarsu *et al.,* 1997; Radhakrishna *et al.,* 1997; Zhao *et al,.* 2002; Galjaard *et al.,* 2003). To date, two

genes have been identified for PAP loci. These include GLI3 and HOXA at PAP-A1 (Radhakrishna *et al.,* 1997; Furniss *et al,*. 2007).

Description of the familial studies

Family A

The family resides in Punjab, Pakistan. A three-generation pedigree of the family was constructed by interviewing the elders of the family (Figure 3.1). The information was cross- checked by interviewing several relatives. Six affected (II-3, III-12, III-5, III-8, III-9, and III-1) and six normal subjects (II-7, II-2, II-8, III-7, III-2, and III-4) of the family were physically examined.

All affected subjects have cutaneous fusion between the middle and ring finger or webbing of third, fourth and fifth finger. An autosomal dominant inheritance is most likely (Figure 3.1). Blood samples from six affected and six normal subjects were obtained and processed for genomic DNA extraction.

Family B

The Family B resides in North West Frontier Province (NWFP), Pakistan. A six-generation pedigree of the family was constructed by interviewing the elders of the family (Figure 3.2). The information was cross- checked by interviewing several relatives. Three affected (V-4, V-7, and VI-3) and ten normal subjects (III-4, IV-16, IV-2, IV-10, V-3, V-5, V-1, V-2, VI-2, and VI-4) of the family were physically examined. Two affected subjects (V-4, V-7) have postaxial polydactyly along with skeletal deformity (bended feet) and speech problem while one subject (VI-3) was having Mental Retardation. An autosomal dominant inheritance is most likely (Figure 3.2). Blood samples from three affected and ten normal subjects were obtained and processed for genomic DNA extraction.

Linkage Studies

Exclusion of Loci for Syndactyly type I, II, III, IV and V

Family A was evaluated for the possibility that the phenotype is linked to the known loci for Syndactyly, named 2q34-q36 (SD1), 3p21.31, 2q31, 22q13.3, 6q22-q23, 7q36, 2q31–q32 (Sarfarazi *et al.,* 1995; Gladwin *et al.,* 1997; Bosse *et al.,* 2000; Paznekas *et al.,* 2003; Malik *et al.*, 2005; Sato *et al.,* 2007; Zhao *et al.,* 2007). No evidence of linkage was witnessed for all loci. Two point linkage

analysis yielded significant negative LOD scores at θ = 0.0 for all loci. The results excluded all critical regions for syndactylies type I, II, III, IV and V flanked by markers D2S1776-D2S1391, D3S2409, D22S445, D6S474, D7S1823- D7S559.

Exclusion of Loci for Postaxial Polydactyly Type I, II, III and IV

Family B was evaluated for the possibility that the phenotype is linked to the known loci for Postaxial polydactyly, named 7p13, 13q21-q32, 19p13.2-p13.1, 7q22 (Akarsu *et al.,* 1997; Radhakrishna *et al.,* 1997; Zhao *et al.,* 2002; Galjaard *et al.,* 2003; Furniss *et al.,* 2007).

Results of two point linkage analysis provided strong evidence that polydactyly locus is linked to marker on chromosome 19p13.2-13.1. A maximum LOD Score calculated was 1.24 at recombination fraction of 0.0 and penetrance of 1.00 at D19S433 locus.

Candidate Disease Gene Identification

Lists of Candidate Disease genes were generated in mapped loci of Family B by using web based tools of G2D (Perez-Iratxeta *et al.,* 2005), PROSPECT and SUSPECT (Adie *et al.,* 2006), GeneDistiller (Seelow *et al.,* 2008), GeneWanderer (Kohler *et al.,* 2008) and PosMed (Yoshida *et al.,* 2009). The list was refined to best candidates depending upon their similarity with limb anomalies associated genes GLI3, HOXD13 and FBLN1. The candidates were ranked by using weighted score of SUSPECT. The list of selected PAP-3 associated candidates is presented in Table 3.1.

Discussion

In contrast to the European population, the Pakistani population has stable communities, large sib-ships and a high consanguinity rate. Marriages within families are strongly favored due to the existence of various linguistic and racial levels, Cultural and economic differences.

In present study, two large inbred Pakistani families (Figure 3.1 and Figure 3.3) with unidentified limb anomalies and several affected individuals were ascertained from Punjab and NWFP, Pakistan, respectively. We reasoned that these large families may provide an excellent opportunity to localize the limb malformation in the human genome, to identify the primary gene and hence, to get to know the

causal patho-mechanisms of the malformations. Therefore, these families were visited at their places of residence in order to get permission from the families to conduct a clinical and molecular study. In each family affected and normal subjects were examined to categorize the limb malformation. In order to infer the correct lineage and the inheritance pattern, an extended pedigree was drawn in each case with the help of the senior family members. Information was obtained about intermarriages, deceased and related defects.

Syndactyly observed in Family A shows typical feature of Syndactyly i.e. complete or partial Webbing of 3rd and 4th fingers or webbing of third, Fourth and fifth finger. Family was evaluated for the possibility that the phenotype is linked to all known loci for Syndactyly, but no linkage was witnessed and also, significant negative LOD Score excluded all critical regions involved in different types of Syndactyly. This indicated that phenotype may be linked to some novel loci.

Postaxial Polydactyly observed in Family B clinically fits PAP-A3, because all patients had one extra finger and/or toe on the ulnar or fibular side of the hands and feet, respectively. Linkage analysis demonstrated that PAP-A3 locus was assigned to chromosome 19p13.2-13.2. The result confirmed the previous assignment of PAP-A3 (Zhao *et al.,* 2002), although this data do not contribute in narrowing the critical region. Gene(s) responsible for PAP-A3 has not been identified yet.

Success in identifying causative genes for hereditary disorders has lifted the hopes up that such genes can be used as target for new drugs, and also it could improve the diagnostic and prognostic information (Yang *et al.,* 2003). However genome-wide techniques are expensive and also generate large sets of candidate genes (Risch, 2000). An important role of bioinformatics approach is in analyzing these large data sets and generates a subset of most likely candidates. Many computational approaches have been designed for this purpose which extracts this information by mining variety of data sources having sequence data, biological information, functional information and expression data for candidate genes (Kanehisa and Bork, 2003; Stein, 2003).

In this study, computational tools i.e G2D (Perez-Iratxeta *et al.,* 2005), SUSPECT, PROSPECTR (Adie *et al.,* 2006), GeneDistiller (Seelow *et al.,* 2008), GeneWanderer (Kohler *et al.,* 2008) and PosMed (Yoshida *et al.,* 2009) have been

employed to gather a subset of genes that are positionally linked to and could be causing Postaxial Polydactyly (PAP-3). The criteria for gene finding in SUSPECT and PROSPECTR are similarity of genes with GLI3, HOXD13 and FBLN1 and their weighted Score. While in G2D are as follow: The source of protein, homolog to translated candidate genomic interval, must be Homo sapiens; the locus of homolog protein should be preferably from already reported candidate disease genes. If a gene follows these criteria then the next step is to check the information regarding biological, functional and expression data. Gene Ontology Score (GO Score), Relative Score (R-Score), Energy Value (E-Value) were also checked. In GeneWanderer, GeneDistiller and PosMed the criteria was to select genes having gene-phenotype associations, gene expression patterns and protein-protein interactions.

The region 19p13.2-13.1 codes for more than 400 genes. A list of all cloned and identified genes from this interval was obtained from Ensemble database. To identify the best likely gene, SUSPECT and PROSPECTR were used which produced a list of more than 300 genes, G2D, GeneWanderer and GeneDistiller gave 100 candidates, PosMed produced 40 candidates. In SUSPECT Search results were limited by selecting only the genes related to Marker D19S221, the only reported marker in this region with maximum LOD Score for PAP-3. GeneWanderer and GeneDistiller generated list of candidates depending upon chromosomal position of PAP-3 loci. The list was further narrowed down to best candidates for the genes meeting the above mentioned criteria. The strong candidates, based on above analysis for PAP-A3 are presented in Table 3.1.

The present study has been confirmed that the Zinc finger containing genes, Krupple Family members, are strong disease candidate genes for PAP-3 as proposed by Zhao *et al.,* 2002 that cluster of zinc fingers containing genes present in this area can be considered as strong candidates for PAP. Following Zinc finger containing genes are considered as candidates: Zinc finger protein 136 (ZNF136); Zinc finger protein 443 (Kruppel-type zinc finger protein ZK1; ZNF443); zinc finger protein 563 (ZNF563); Zinc finger protein 442 (ZNF442); Zinc finger protein 44 (Zinc finger protein KOX7; Gonadotropin inducible transcription repressor-2(GIOT-2); ZNF44). These genes share a strong homology with GLI3 e.g. ZNF136 shares transcription from Pol II promoter and Zn-finger, C2H2 type

domain with GLI3, while others i.e. ZNF443, ZNF563, ZNF442, ZNF44 share shares only Zn-finger, C2H2 type domain with GLI3, Hence considered as strong candidates.

Depending upon the weighted score given in SUSPECT, Erythroid krueppel-like transcription factor (EKLF; Kruppel like factor 1(KLF1)) is considered as next strongest candidate, as it also shares transcription from Pol II promoter and Zn-finger, C2H2 type domain with GLI3. Nuclear Factor I/X (CCAAT-binding transcription factor; NFIX) is preferred as candidate gene as it shares transcription from Pol II promoter with GLI3 and HOXD13, where HOXD13 has been reported to be involve in Synpolydactyly, type II, Synpolydactyly, Brachydactyly, Limb malformation.

Evolutionarily Conserved Signaling Intermediate in Toll pathway (ECSIT homolog (Drosophila); ECSIT) functions as an essential component in two important signal transduction pathways and establishes a novel role for Ecsit as a cofactor for Smad proteins in the bone morphogenetic protein signaling pathway (Xiao *et al.,* 2003), thus regarded as strong candidate. JUN D proto-oncogene (JUND) is another candidate for PAP as it shares transcription from Pol II promoter with GLI3 and HOXD13 and Orphan nuclear receptor, NOR1 type domain with HOXD13 and also this is the only gene selected by all softwares.

Following genes are also considered for their candidature as UV excision repair protein RAD23 homolog A (RAD23A) shares nucleotide-excision repair with GLI3; Transportin 2 (TNPO2) shares protein-nucleus import with GLI3; Arsenical pump-driving ATPase (ASNA-I) shares P-P-bond-hydrolysis-driven transporter activity with GLI3 shares anion transport with FBLN1, FBLN1 has been implicated in Synpolydactyl.

I believe that the genes presented in this study may serve as starting candidates and may be analyzed for identifying molecular mechanism for specific PAP-A3 Phenotypes in parallel with attempts to further narrow down the suspected regions by genetic linkage analysis.

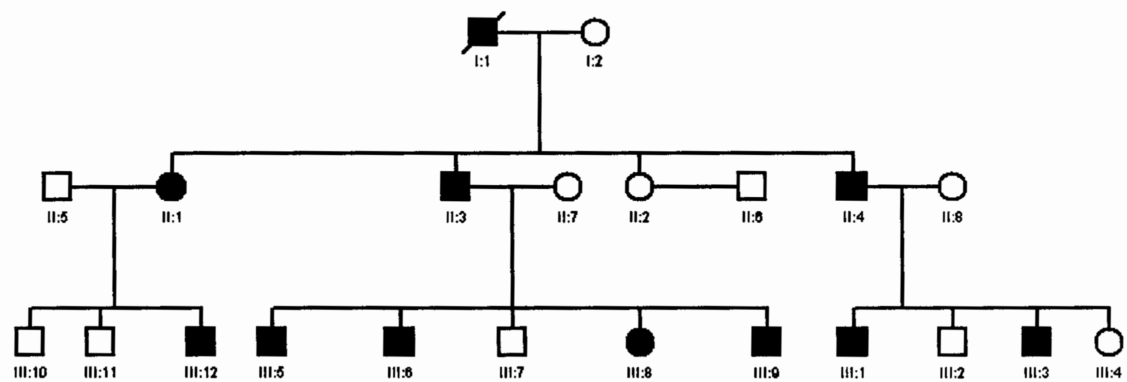


Figure 3.1: Pedigree of family A with Syndactyly.

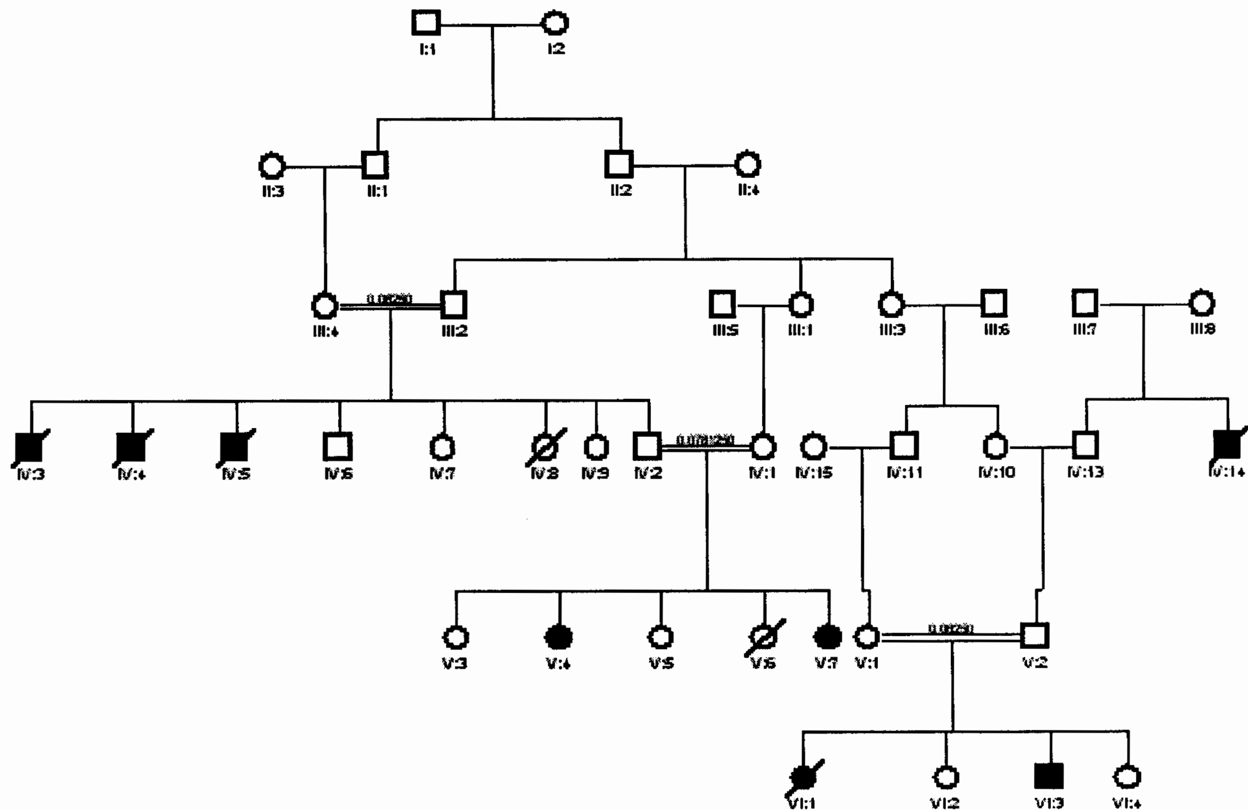


Figure 3.4: Pedigree of family B with Postaxial Polydactyly and Mental Retardation.

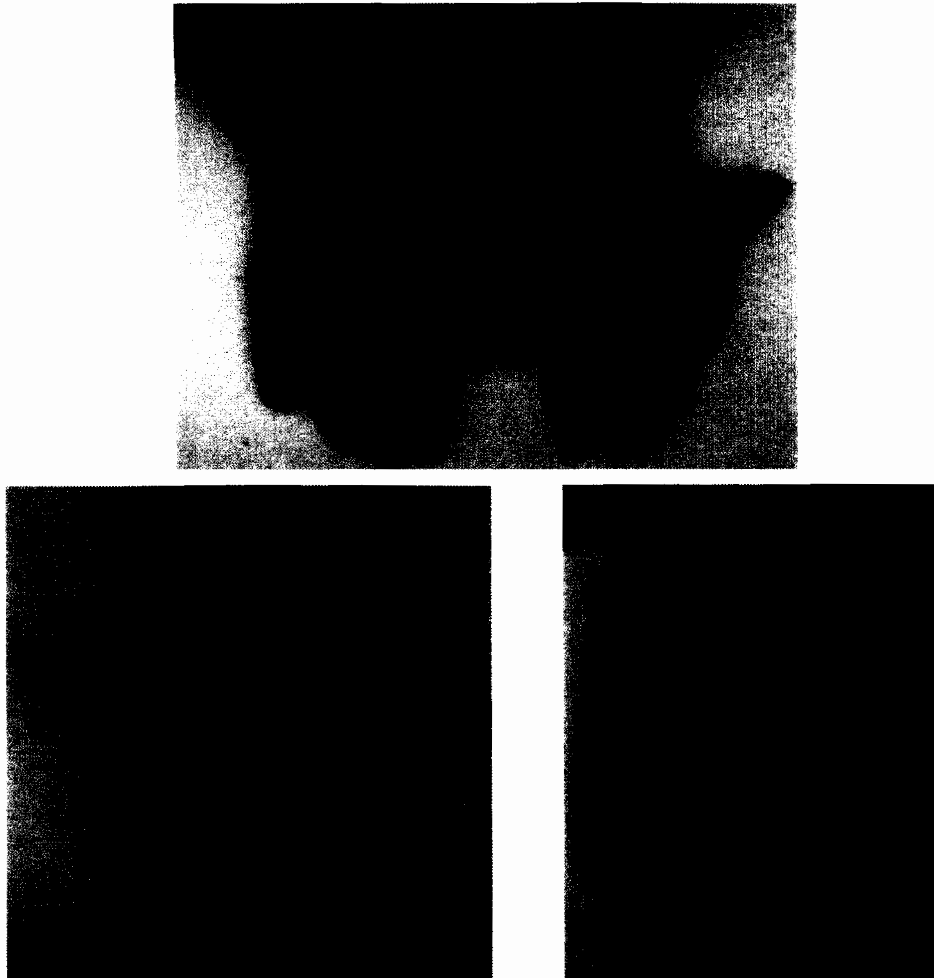
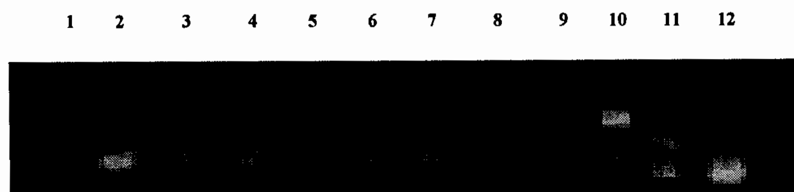


Figure 3.5: An affected Individual (IV-4) from family B with Postaxial Polydactyly.



Family A

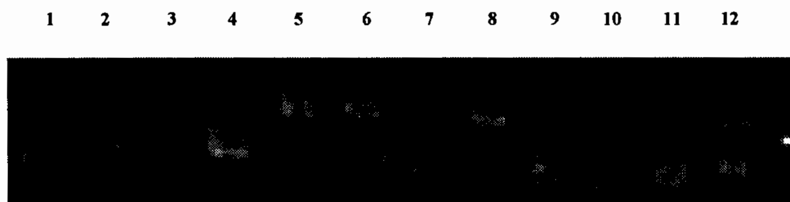
Lane 1. II-3 Lane 5.III-9 Lane 9. III-4

Lane 2. II-7 Lane 6.III-7 Lane 10.III-1

Lane 3.III-5 Lane 7. II-8 Lane 11. II-2

Lane 4.III-8 Lane 8.III-2 Lane 12.III-12

Figure 3.6: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D22S445 at 45.22 cM on chromosome 22q13.3, of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family A

Lane 1. II-3 Lane 5.III-9 Lane 9. III-4

Lane 2. II-7 Lane 6.III-7 Lane 10.III-1

Lane 3.III-5 Lane 7. II-8 Lane 11. II-2

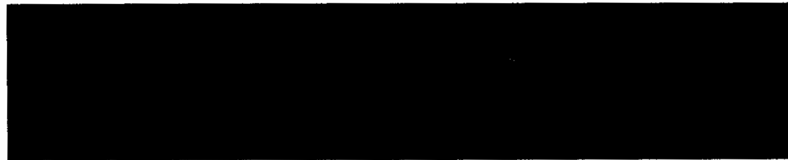
Lane 4.III-8 Lane 8.III-2 Lane 12.III-12

Figure 3.7: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D6S474 at 123.8 cM on chromosome 6q22-q24 of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

Family A

Lane 1. II-3	Lane 5.III-9	Lane 9. III-4
Lane 2. II-7	Lane 6.III-7	Lane 10.III-1
Lane 3.III-5	Lane 7. II-8	Lane 11. II-2
Lane 4.III-8	Lane 8.III-2	Lane 12.III-12

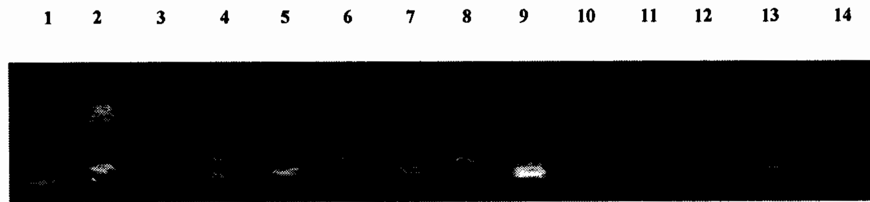
Figure 3.8: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D2S1776 at 168.1 cM on chromosome 2q31–q32 of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family A

Lane 1. II-3	Lane 5.III-9	Lane 9. III-4
Lane 2. II-7	Lane 6.III-7	Lane 10.III-1
Lane 3.III-5	Lane 7. II-8	Lane 11. II-2
Lane 4.III-8	Lane 8.III-2	Lane 12.III-12

Figure 3.9: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D6S1040 at 128.2 cM on chromosome 6q22-q24 of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family B

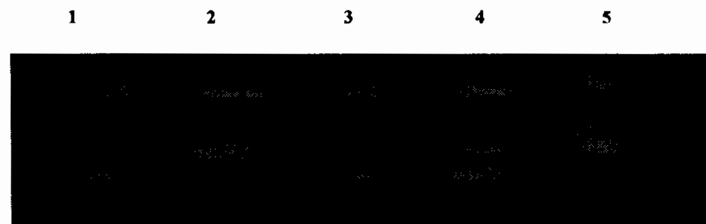
Lane 1. III-4 Lane 5.V-3 Lane 9. IV-10 Lane 13.VI-3

Lane 2.IV-1 Lane 6.V-4 Lane 10.V-2 Lane 14.VI-4

Lane 3.IV-2 Lane 7.V-5 Lane 11.V-1

Lane 4.IV-6 Lane 8.V-7 Lane 12.VI-2

Figure 3.10: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S433 at 37.8 cM on chromosome 19p13.2-p13.1 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

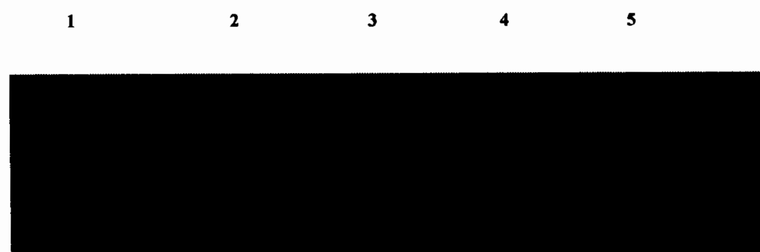


Family B

Lane 1.IV-1 Lane 3.V-4 Lane 5.V-6

Lane 2.V-3 Lane 4.V-5

Figure 3.11: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D7S1799 at 115.6 cM on chromosome 7q22 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

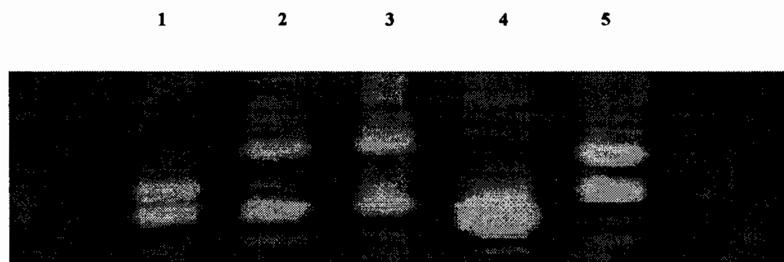


Family B

Lane 1.IV-1 Lane 3.V-4 Lane 5.V-6

Lane 2.V-3 Lane 4.V-5

Figure 3.12: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S586 at 11.3 cM on chromosome 19p13.2-p13.1 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

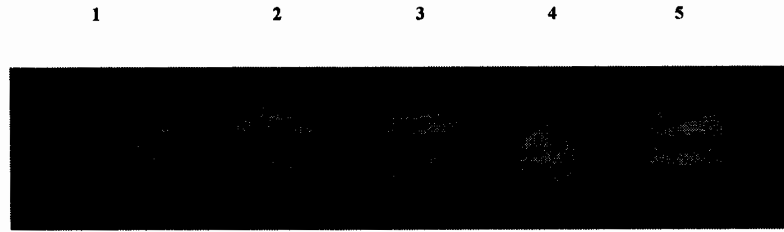


Family B

Lane 1.IV-1 Lane 3.V-4 Lane 5.V-6

Lane 2.V-3 Lane 4.V-5

Figure 3.13: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D7S1818 at 62 cM on chromosome 7p13 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

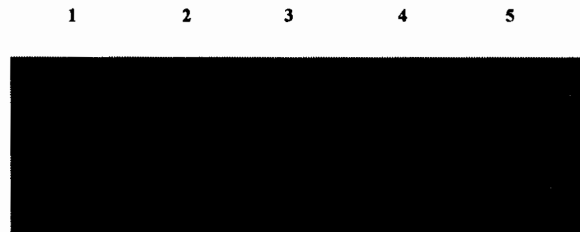


Family B

Lane 1.IV-1 Lane 3.V-4 Lane 5.V-6

Lane 2.V-3 Lane 4.V-5

Figure 3.14: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D7S3046 at 67.1 cM on chromosome 19p13.2-p13.1 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family B

Lane 1.IV-1 Lane 3.V-4 Lane 5.V-6

Lane 2.V-3 Lane 4.V-5

Figure 3.15: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D13S317 at 85.1 cM on chromosome 7p13 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

Table 3.1: Two-point LOD scores between the phenotype and Postaxial Polydactyly markers on chromosome 7p, 7q, 13q, and 19p

Locus	Cytogenetic Location	Markers	Distance (cM)	Recombination Fraction				
				0.1	0.2	0.3	0.4	0.5
PAP-A1	7p13	D7S3046	67.1	-5.07	-3.45	-2.33	-1.41	-0.01
		D7S1818	62.0	-5.21	-3.48	-2.40	-1.47	-0.08
PAP-A2	13q21-q32	D13S317	85.1	-7.43	-5.11	-3.47	-0.43	-0.11
		D13S793	95.8	-6.47	-4.80	-2.77	-1.89	-0.11
PAP-A3	19p13.2-p13.1	D19S586	11.3	-0.70	-0.14	-0.63	-0.71	-0.15
		D19S433	37.8	1.24	0.81	0.41	0.11	0.00
PAP-A4	7q22	D7S1799	115.6	-0.31	-0.09	0.03	0.04	0.01

Table 3.2: Analysis of Selected PAP-3 Candidate Genes by using G2D, SUSPECT, PROSPECTR, Gene Distiller and GeneWanderer.

#	Gene	Description	Chromosomal Location	Similarity	Weighted Score	R-Score	GO Score	P Value
1	RAD23A	UV excision repair protein RAD23 homolog A	12,917,654	GLI3	47.71	0.242851	0.000170	-
2	TNPO2	Transportin 2	12,671,017	GLI3	45.81	0.244290	0.000170	-
3	ASNA-I	Arsenical pump-driving ATPase	12,709,306	GLI3 FBLN1	44.27	-	-	$5.28E^{-4}$
4	KLF1	Erythroid krueppel-like transcription factor	12,856,239	GLI3	29.92	-	-	-
5	ZNF136 ZNF443 ZNF563 ZNF442 ZNF44	Zinc finger protein 136 Zinc finger protein 443 Zinc finger protein 563 Zinc finger protein 442 Zinc finger protein 44	12,156,433 12,401,521 12,289,308 12,321,515 12,214,623	GLI	25.83 24.04 23.91 23.20 23.20	-	-	-
6	ECSIT	Evolutionarily Conserved Signaling Intermediate in Toll pathway	11,477,745	-	11.03	0.015131	0.000521	-
7	NFIX	Nuclear Factor I/X	12,996,459	GLI3 HOXD13	9.95	0.002589	0.000521	-
8	JUND	JUN D proto-oncogene	18,252,251	GLI3 HOXD13	5.42	0.022783	0.000521	$1.70E^{-2}$

Table 3.3: List of Genes Selected by each Method.

#	Gene	Description	SUSPECT	PROSPECTR	G2D	Gene Distiller	GeneWanderer	PosMed
1	RAD23A	UV excision repair protein RAD23 homolog A	√	√	√	√	√	×
2	TNPO2	Transportin 2	√	√	√	√	√	×
3	ASNA-I	Arsenical pump-driving ATPase	√	√	×	√	√	√
4	KLF1	Erythroid krueppel-like transcription factor	√	√	×	√	√	×
5	ZNF136 ZNF443 ZNF563 ZNF442 ZNF44	Zinc finger protein 136 Zinc finger protein 443 Zinc finger protein 563 Zinc finger protein 442 Zinc finger protein 44	√	√	×	√	√	×
6	ECSIT	Evolutionarily Conserved Signaling Intermediate in Toll pathway	√	√	√	×	×	×
7	NFIX	Nuclear Factor I/X	√	√	√	√	√	×
8	JUND	JUN D proto-oncogene	√	√	√	√	√	√

MUSCULAR DYSTROPHY (MD)

Muscular Dystrophies are a group of genetic diseases of the muscular system illustrated by weakness of the muscles of the body. It is progressive and remission does not occur (Emery, 2008). With time the weakness increase and affects different muscles. MD is caused by missing or incorrect genetic information in the body's cells. As a result body is unable to make the proteins needed to build and maintain healthy muscles (Johanson, 2008). Nine types of MD have been described yet.

BMD shows X-linked inheritance and was found to be linked with chromosomal region Xp21.2 (Becker, 1957; Kingston *et al.,* 1983, 1984, Brown *et al.,* 1985; Fadda *et al.,* 1985; Roncuzzi *et al.,* 1985). It is caused by mutation in the gene encoding dystrophin (Passos-Bueno *et al.,* 1994; Arikawa-Hirasawa *et al.,* 1995; Yagi *et al.,* 2003; Tuffery-Giraud *et al.,* 2005).

Different types of CMDs have been described. MDC1A is reported to be caused by primary deficiency of LAMA2 due to mutations in the LAMA2 gene and is linked to chromosomal region of 6q2 (Hillaire *et al.,* 1994). MDC1B is caused by secondary deficiency of LAMA2 in muscle and is linked to chromosome 1q42 (Muntoni *et al.,* 1998; Brockington *et al.,* 2000). MDC1C is caused by mutation in the gene, encoding the FKRP, located on chromosome 19q13.3 (Brockington *et al.,* 2001; Louhichi *et al.,* 2004; MacLeod *et al.,* 2007). MDC1D is caused by mutation in the gene, encoding LARGE, located on chromosomal region 22q12.3-q13.1 (Longman *et al.,* 2003). RSMD1 is caused due to mutations in the SEPN1 gene (Moghadaszadeh *et al.,* 1998). UCMD is caused by recessive mutations in any of 3 genes encoding the subunits of COL6A1; COL6A2; and COL6A3 (Mercuri *et al.,* 2002; Pan *et al.,* 2003; Baker *et al.,* 2005; Giusti *et al.,* 2005; Lampe *et al.,* 2005; Angelin *et al.,* 2007; Kawahara *et al.,* 2007). van Reeuwijk *et al.,* (2006) identified compound heterozygous mutations in the POMT1 gene responsible for WWS (Muscular Dystrophy, Congenital, Plus Mental Retardation, Included). Gene for FCMD was localized on chromosome 9q31-q33 (Toda *et al.,* 1996) and caused by mutation in the gene encoding FKTN (Kobayashi et al., 1998; Kondo-Iida et al., 1999). Cormand *et al.,* (1999) reported linkage for MEB gene on chromosome1p34-p32. Loss-of-function mutations in the POMGNT1 gene are responsible for MEB (Yoshida *et al.,* 2001; Taniguchi *et al.,* 2003; Vervoort *et al.,* 2004).

DMD is caused by mutation in the gene encoding DMD which was found to be linked on chromosomal region Xp21 (Lindenbaum *et al.,* 1979; Zneimer *et al.,* 1993). EDMD is comprises of 2 Emery-Dreifuss syndromes caused by mutation in the LMNA one displays similar features to EDMD and is autosomal dominant (EDMD2), and the other appears to lack cardiac features and is autosomal recessive (EDMD3). Emery-Dreifuss Muscular Dystrophy, X-Linked (EDMD) is inherited as an X-linked recessive disorder, although autosomal dominant mode of inheritance has also been reported (Rudenskaya *et al.,* 1994). Boswinkel *et al.,* (1985) reported linkage for EDMD on chromosomal region Xq28. This region was further refined by Thomas *et al.,* (1986), Romeo *et al.,* (1988), Consalez *et al.,* (1991) and Yates *et al.,* (1993). Mutation causing EDMD has been identified in the gene encoding EMD (Hoeltzenbein *et al.,* 1999; Ben Yaou *et al.,* 2007). Bonne *et al.,* (1999) mapped the locus for EDMD2 on chromosome 1q11-q23. Mutations causing EDMD2 in the LMNA gene have been identified (Bonne *et al.,* 1999; Muchir *et al.,* 2000). Raffaele di Barletta *et al.,* (2000) identified heterozygous mutations causing EDMD3 in the LMNA gene located on chromosome 1q21.2.

Mathews *et al.,* (1991) defined the location of FSHD as 4q35. By linkage analysis of a homogeneous group of Oculopharyngeal Muscular Dystrophy (OPMD) families, Brais *et al.,* (1995) identified a putative disease locus within a 5-cM segment of chromosome 14q11.2-q13.

The classic form of Myotonic dystrophy (DM), DM1 is caused by an expanded CTG repeat in the DMPK on 19q13 (Eiberg *et al.,* 1983). DM2 is caused by expansion of a CCTG repeat in intron 1 of the ZNF9 on 3q13.3-q24 (Ranum *et al.,* 1998).

DYSF is considered as a prime candidate gene for all forms of DMAT, WDM and MM (Liu *et al.,* 1998; Ahlberg *et al.,* 1999) and all forms are localized on chromosome 2p13.3-p13.1 (von Tell *et al.,* 2003).

Several forms of autosomal dominant LGMD has been identified which include LGMD1A, localized on 5q31 (Speer *et al.,* 1992), with autosomal dominant inheritance, caused by mutation in the gene encoding TTID (Hauser *et al.,* 2000, 2002); LGMD1B, mapped on 1q21.2 (Van der Kooi *et al.,* 1997), shows autosomal dominant inheritance (Van der Kooi *et al.,* 1996, 1997), caused by mutation in the gene encoding LMNA (Muchir *et al.,* 2000; van Engelen *et al.,* 2005); LGMD1C,

caused by mutation in the CAV3 which is located on 3p25 and shows autosomal dominant inheritance (Minetti *et al.,* 1998; McNally *et al.,* 1998; Betz *et al.,* 2001; de Paula *et al.,* 2001); LGMD1D, localized on 7q (Speer *et al.,* 1999), shows autosomal dominant inheritance (Chutkow *et al.,* 1986); LGMD1E, localized on 6q23 (Messina *et al.,* 1997); LGMD1F , localized on 7q32.1-q32.2 (Gamez *et al.,* 2001, Palenzuela *et al.,* 2003); LGMD1G, localized on chromosome 4q21 (Starling *et al.,* 2004); LGMD2A, localized on 15q15.1-q21.1, with autosomal recessive inheritance and is caused by mutation in the gene encoding the CAPN3 (Passos-Bueno *et al.,* 1993; Fougerousse *et al.,* 1994; Allamand *et al.,* 1995); LGMD2B, localized on 2p13.3-p13.1 (Bashir *et al.,* 1996), with autosomal recessive inheritance (Bashir *et al.,* 1994), caused by mutations in the gene encoding the skeletal muscle protein DYSF (Bashir *et al.,* (1998); LGMD2C, localized on 13q12 (Ben Othmane *et al.,* 1992) with autosomal recessive inheritance (Hazama *et al.,* 1979) and caused by mutation in the SGCG (Noguchi *et al.,* (1995); LGMD2D, localized on 17q12-q21.33 with autosomal recessive inheritance and caused by mutation in the SGCA (Roberds *et al.,* 1994; Passos Bueno *et al.,* 1995; Piccolo *et al.* 1995; Trabelsi *et al.* 2008); LGMD2E, localized on chromosome 4q12, shows autosomal recessive inheritance and is caused by mutation in the gene encoding SGCB (Lim *et al.,* 1995; Bonnemann *et al.,* 1996; Trabelsi *et al.,* 2008); LGMD2F, localized on 5q33-q34, shows autosomal recessive inheritance and caused by mutation in the SGCD (Nigro *et al.,* 1996; Passos-Bueno *et al.,* 1996 Moreira *et al.,* 1998; Trabelsi *et al.,* 2008); LGMD2G, linked to region on chromosome 17q11-q12 and is caused by mutation in the gene encoding TCAP (Moreira *et al.,* 1997, 2000); LGMD2H, shows autosomal recessive inheritance and caused by mutation in the gene encoding TRIM32 on chromosome 9q31-q34.1 (Weiler *et al.,* 1998; Frosk *et al.,* 2002); LGMD2I, localized on 19q13.3, shows autosomal recessive inheritance, caused by mutation in the gene encoding FKRP (Driss *et al.,* 2000, Brockington *et al.*, 2001; de Paula *et al.*, 2003; Harel *et al.*, 2004; Frosk *et al.*, 2005); LGMD2J, localized on 2q31 and caused by homozygous mutation in the TTN (Udd *et al.,* 1992, Haravuori *et al.*, 1997, 1998, 2002); LGMD2K, caused by mutation in the POMT1 on 9q34 (Dincer *et al.,* 2003, Balci *et al.,* 2005; D'Amico *et al.,* 2006); LGMD2L, localized on 11p13-p12 (Jarry *et al.,* 2007).

Description of the Familial Studies

Family C

The family C resides in Punjab, Pakistan. A four-generation pedigree of the family was constructed by interviewing the elders of the family (Figure 4.1). The information was cross- checked by interviewing several relatives. Four affected (IV-6, IV-7, IV-8 and IV-1) and six normal subjects (III-2, III-1, IV-11, IV-10, IV-2, and IV-3) of the family were physically examined.

All affected subjects have week muscles of lower limbs, bended feet and eventual loss of ability to walk. An X-linked disorder is most likely (Figure 4.1). Blood samples from four affected and six normal subjects were obtained and processed for genomic DNA extraction.

Family D

The family D resides in Punjab, Pakistan. A four-generation pedigree of the family was constructed by interviewing the elders of the family (Figure 4.2). The information was cross- checked by interviewing several relatives. Two affected (IV-10, IV-11) and four normal subjects (III-2, IV-8, IV-9 and IV-12) of the family were physically examined.

All affected subjects have week muscles of lower limbs, bended feet and eventual loss of ability to walk (Figure 4.2). Blood samples from four affected and six normal subjects were obtained and processed for genomic DNA extraction.

Linkage Studies

Exclusion of Loci for Muscualr Dystrophies

Family C and D were evaluated for the possibility that the phenotype is linked to the known loci for Muscualr Dystrophy. Two point linkage analysis yielded significant negative LOD scores at $\theta = 0.0$ for all loci. These results excluded all critical regions for Muscualr Dystrophies, flanked by respective markers (Table 2.3).

Docking Analysis

In the present study, Triterpene antioxidants, including Ganoderic acids A, B, C, and D; Ganoderol A and B and Ganodermanontriol found in Reishi extracts, have been analyzed computationally and compared with available supplements for muscular

dystrophy i.e. Creatine and Coenzyme Q10. The docking energies, No of hydrogen bonds formed, number of clusters, Intermolecular Energies, Internal Energies of Ligand and Torsional Free Energies are presented in Table 4.1 and Table 4.2.

Discussion

In the present study, two inbred Pakistani families (Figure 4.1 and Figure 4.2) with Muscle deformities and several affected individuals were ascertained from Punjab. We reasoned that these families may provide an excellent opportunity to localize the Muscular Dystrophy in the human genome, to identify the primary gene and hence, to get to know the causal patho-mechanisms of the malformations. Therefore, these families were visited at their places of residence in order to get permission from the families to conduct a clinical and molecular study. In each family affected and normal subjects were examined to categorize the Muscle deformities. In order to infer the correct lineage and the inheritance pattern, an extended pedigree was drawn in each case with the help of the senior family members. Information was obtained about intermarriages, deceased and related defects.

Family C and D shows typical feature of Muscular Dystrophy i.e. Progressive weakness of the muscles of the body, Musculae weekness in lower limbs and eventual loss of walking ability. Families were evaluated for the possibility that the phenotype is linked to all known loci for Muscular Dystrophy, but no linkage was witnessed and also, significant negative LOD Score excluded all critical regions involved in different types of Muscular Dystrophy.

Laboratory techniques, though, one of the most direct and reliable approaches for evaluating any drug candidature chemical compound but they are insufficient, to meet the daunting requirements of accurate risk assessment and informed decision making, as they are costly and can take four to five years to complete. But bioinformatics approaches are serving as one of the major player in field of drug industry. By using the power of Bioinformatics, a wealth of data is being generated that can be used to identify and select suitable targets for new drugs. Structure based or rational drug discovery has resulted in a number of drugs in the market and many more are under development (Rastogi *et al.,* 2004).

In the present study, Triterpene antioxidants, including ganoderic acids A, B, C, and D; ganoderol A and B and ganodermanontriol found in Reishi extracts, have been analyzed computationally and compared with available supplements for muscular dystrophy i.e. Creatine and Coenzyme Q10. Docking tools used are AutoDock and Hex. The lowest docking energies, as calculated by AutodDock, are of Ganoderic acid A, Ganoderol A, Ganoderic acid B and Ganoderol B. Their docking energies are as follow: -9.68 kcal/mol, -9.67 kcal/mol, -9.40 kcal/mol and -9.11 respectively. The docking energies of supplements i.e. Creatine and Coenzyme Q10 are -4.74 kcal/mol and -7.95 kcal/mol respectively. The Total energies of Receptor-Ligand Complex calculated by Hex are: -238.11 for Dystrophin-Ganoderic Acid A complex; -243.07 for Dystrophin-Ganoderic Acid B complex; -219.50 for Dystrophin- Ganoderol A complex; 0.00 for Dystrophin- Ganoderol B complex; -325.61 for Dystrophin-CoenzymeQ10 complex and -115.69 for Dystrophin- Creatine complex (Figure 4.27-4.32).

Analysis of AutoDock and Hex results shows that Ganodric Acid A has lowest docking energy than all of the rest mentioned compounds and conformational analysis shows that it forms three hydrogen bonds with receptor protein at TRP118 and ASN122 (Figure 4.16). Information regarding hydrogen bond formation is the strong indication that Ligand is going to be covalently attached with receptor. Ganoderol A which has almost equal docking energy to Ganoderic acid A i.e. -9.67 kcal/mol forms no hydrogen bond with the receptor (Fugure 4.20) so the above calculated energy is not of the Ligand-receptor complex rather it is only a garbage value. Ganodric Acid B forms one hydrogen bond at HIS117 (Figure 4.18) while Ganoderol B forms no hydrogen bond (Fugure 4.22). When Ganoderol A and B were further analysed at other conformations, it was noticed that Ganoderol A was forming hydrogen bond at ASN88 with both 4th and 8th conformation while Ganoderol B was forming hydrogen bond at ASN87 and TRP118 with 6th and 10th conformation respectively. Creatine forms two hydrogen bonds at HIS117 and ASN86 (Fugure 4.24) while Coenzyme Q10 forms no hydrogen bond at lowest docking energy conformation (Fugure 4.26) rather it forms hydrogen bond at ASN86 and HIS117 with 3rd and 6th conformation. Ganoderol A has the lowest Intermolecular Energy while creatine has the lowest Internal Energy of Ligand and Torsional Free Energy (Table 4.2). There are ten conformational clusters formed with coenzyme Q10 while nine with Ganoderic acid A which is evidence of best docking site within receptor protein.

Comparitve analysis, on the basis of docking energy, number of hydrogen bonds formed and number of clusters, rank Ganodeic Acid A at highest and then Ganoderic Acid B. No doubt the docking energy of creatine is more than coenzyme Q10 but it has the lowest Internal Energy of Ligand and Torsional Free Energy and forms hydrogen bond with best conformation so creatine is ranked higher than coenzyme Q10. As consumption of antioxidants is essential for protection against oxidative stress, which is a major cause of Muscular Dystrophy, Ganoderic acid A and Ganoderic Acid B, because of their antioxidant activity, can be best supplements for treating all Muscular Dystrophies. I also propose that they may help in Muscular Dystrophy especially in treating Duchenne type and Dystrophia Myotonica, by restoring normal translation of mutated genes by avoiding premature termination during translation.

Although computational analysis has favored the consumption of Ganoderic acid A and Ganoderic Acid B for treating Muscular Dystrophy, there is need of clinical trials to be carried out to identify specific Triterpene antioxidants that have good therapeutic effects. As antioxidants are effective against certain free radicals and they tend to locate preferentially in different types of cells and tissues so it is also required to be check that either wide range of antioxidants in low dosage or high doses of one or two of these compounds is beneficial.

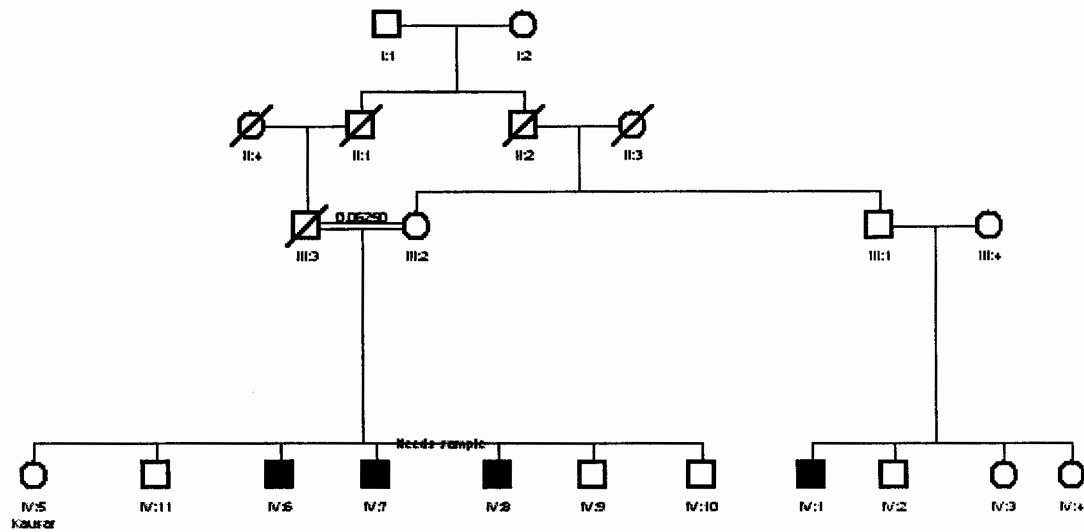


Figure 4.1: Pedigree of family C with Muscular Dystrophy.

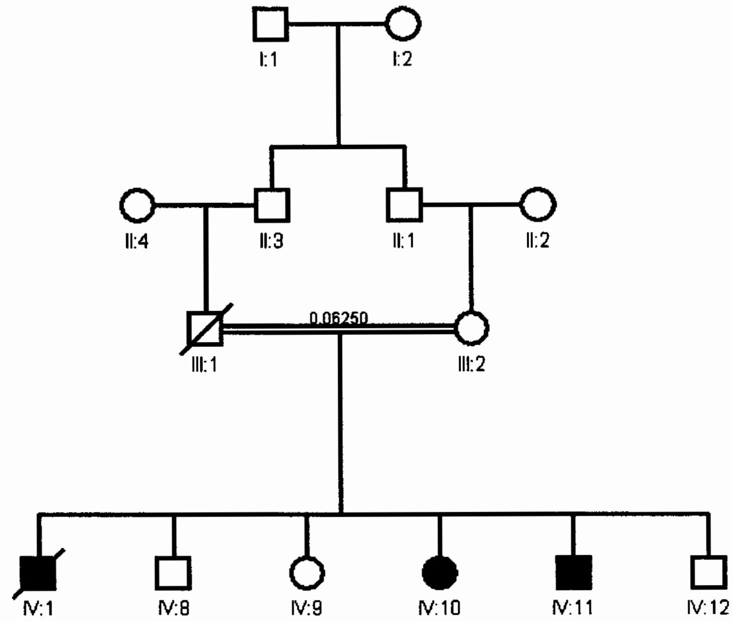


Figure 4.2: Pedigree of family D with Muscular Dystrophy.

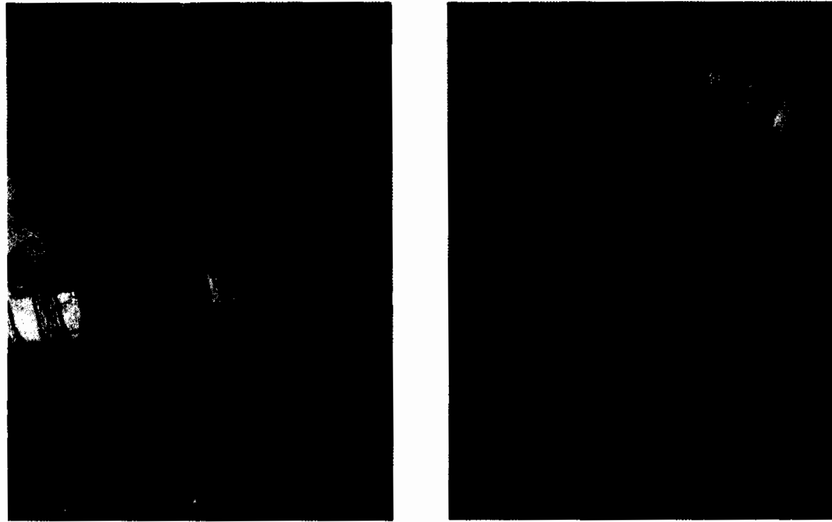


Figure 4.3: An affected Individual (IV-10) from family D with Muscular Dystrophy.

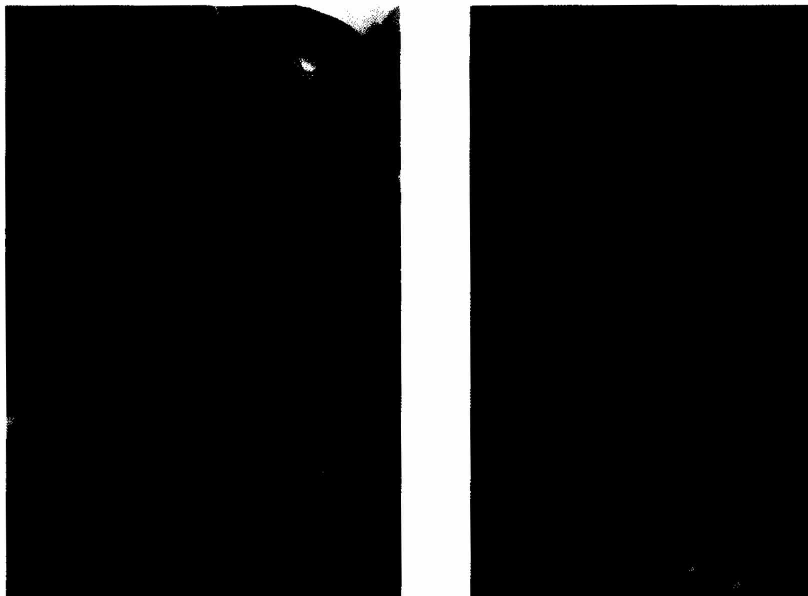


Figure 4.4: An affected Individual (IV-11) from family D with Muscular Dystrophy.

1 2 3 4 5 6 7

Family C

Lane 1. III-2 Lane 3. IV-6 Lane 5. IV-8 Lane 7. IV-1

Lane 2. IV-11 Lane 4. IV-7 Lane 6.IV-10

Figure 4.5: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S178 at 48.5 cM on chromosome 19q13.3 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

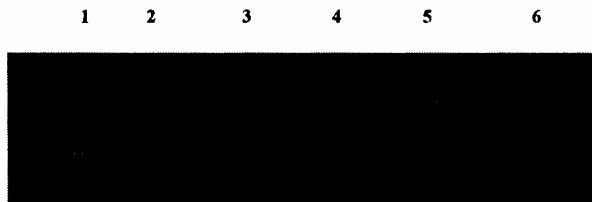
1 2 3 4 5 6

Family C

Lane 1. III-2 Lane 3. IV-6 Lane 5. IV-8

Lane 2. IV-11 Lane 4. IV-7 Lane 6.IV-10

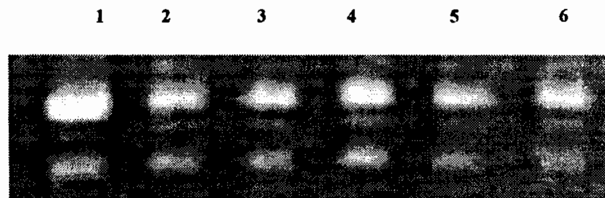
Figure 4.6: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D9S934 at 123.7 cM on chromosome 9q31 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family C

Lane 1. III-2	Lane 3. IV-6	Lane 5. IV-8
Lane 2. IV-11	Lane 4. IV-7	Lane 6.IV-10

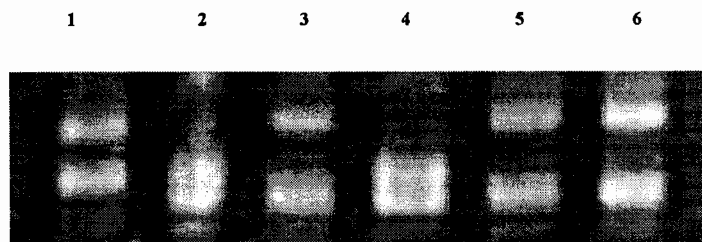
Figure 4.7: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker DXS6810 at 42.7 cM on chromosome Xp21.2 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family C

Lane 1. III-2	Lane 3. IV-6	Lane 5. IV-8
Lane 2. IV-11	Lane 4. IV-7	Lane 6.IV-10

Figure 4.8: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D1S1622 at 32.5 cM on chromosome 1p36-p35 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

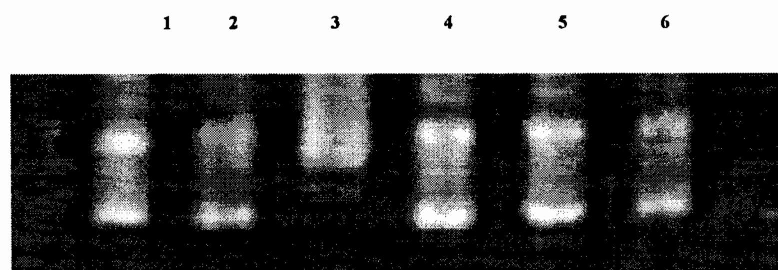


Family C

Lane 1. III-2 Lane 3. IV-6 Lane 5. IV-8

Lane 2. IV-11 Lane 4. IV-7 Lane 6.IV-10

Figure 4.9: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker DXS9908 at 143.9 cM on chromosome Xq28 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

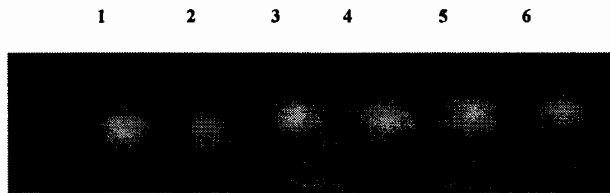


Family C

Lane 1. III-2 Lane 3. IV-6 Lane 5. IV-8

Lane 2. IV-11 Lane 4. IV-7 Lane 6.IV-10

Figure 4.10: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker DXYS154 at 159.9 cM on chromosome Xq28 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

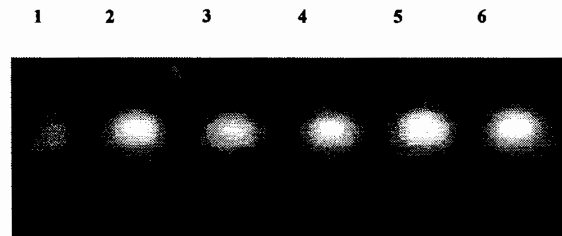


Family D

Lane 1.III-2 Lane 3.IV-9 Lane 5.IV-11

Lane 2.IV-8 Lane 4.IV-10 Lane 6.IV-12

Figure 4.11: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D1S549 at 239.5 cM on chromosome 1q42 of family D. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family D

Lane 1.III-2 Lane 3.IV-9 Lane 5.IV-11

Lane 2.IV-8 Lane 4.IV-10 Lane 6.IV-12

Figure 4.12: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S246 at 57.8 cM on chromosome 19q13.3 of family D. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

1 2 3 4 5 6

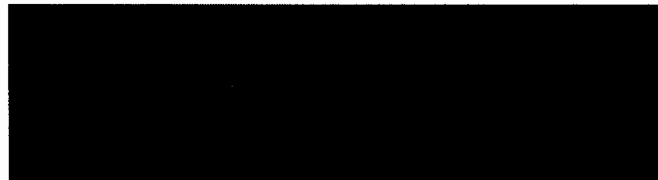
Family D

Lane 1.III-2 Lane 3.IV-9 Lane 5.IV-11

Lane 2.IV-8 Lane 4.IV-10 Lane 6.IV-12

Figure 4.13: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker DXS6810 at 42.7 cM on chromosome Xp21.2 of family D. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

1 2 3 4 5 6



Family D

Lane 1.III-2 Lane 3.IV-9 Lane 5.IV-11

Lane 2.IV-8 Lane 4.IV-10 Lane 6.IV-12

Figure 4.14: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D1S1622 at 32.5 cM on chromosome 1p36-p35 of family D. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

Table 4.1: AutoDock results of Dystrophin protein with all Triterpene antioxidants and commonly used Supplements

No	Compound	No of clusters	Intermolecular Energy kcal/mol	Internal Energy of Ligand kcal/mol	Torsional Free Energy kcal/mol	Reference RMSD	Cluster RMSD	Lowest Docked Energy kcal/mol	Mean Docked Energy kcal/mol
1	Ganoderic Acid A	9	-9.10	-0.58	+1.56	17.27	0.00	-9.68	-9.68
2	Ganoderic Acid B	4	-8.68	-0.72	+1.87	15.42	0.00	-9.40	-8.79
3	Ganoderic Acid C	8	-7.81	+0.02	+1.87	16.29	0.00	-7.79	-7.79
4	Ganoderic Acid D	9	-8.35	-0.22	+1.87	15.51	0.00	-8.57	-8.57
5	Ganoderol A	8	-9.73	+0.06	+1.56	9.91	0.00	-9.67	-9.67
6	Ganoderol B	6	-8.90	-0.21	+1.56	33.08	0.00	-9.11	-9.11
7	Ganodermanontriol	9	-7.14	+0.43	+1.87	35.97	0.00	-6.71	-6.65
8	Creatine	3	-4.70	-0.09	+0.62	38.39	0.00	-4.79	-4.74
9	Coenzyme Q10	10	-9.17	+1.23	+9.65	31.90	0.00	-7.95	-7.95

Table 4.2: Analysis of docking results with selected Triterpene antioxidants and commonly used Supplements

No.	Compound	Best Conformation No.	Lowest Docking Energy	No of Hydrogen Bonds formed at best conformation	Residue which is forming Hydrogen Bond at best conformation	No of Hydrogen Bonds formed at other conformation	Residue which is forming Hydrogen Bond at other conformation
1	Ganoderic Acid A	7th	-9.68	3	TRP118(1) ASN122(2)	-	-
2	Ganoderic Acid B	9th	-9.40	1	HIS117	-	-
3	Ganoderol A	9th	-9.67	nil	-	1 at Conformation 4 1 at Conformation 8	ASN88 ASN88
4	Ganoderol B	2nd	-9.11	nil	-	1 at Conformation 6 1 at Conformation 10	ASN87 TRP118
5	Creatine	8th	-4.79	2	HIS117 ASN86	-	-
6	Coenzyme Q10	1st	-7.95	nil	-	1 at Conformation 3 1 at Conformation 6	ASN88 HIS117

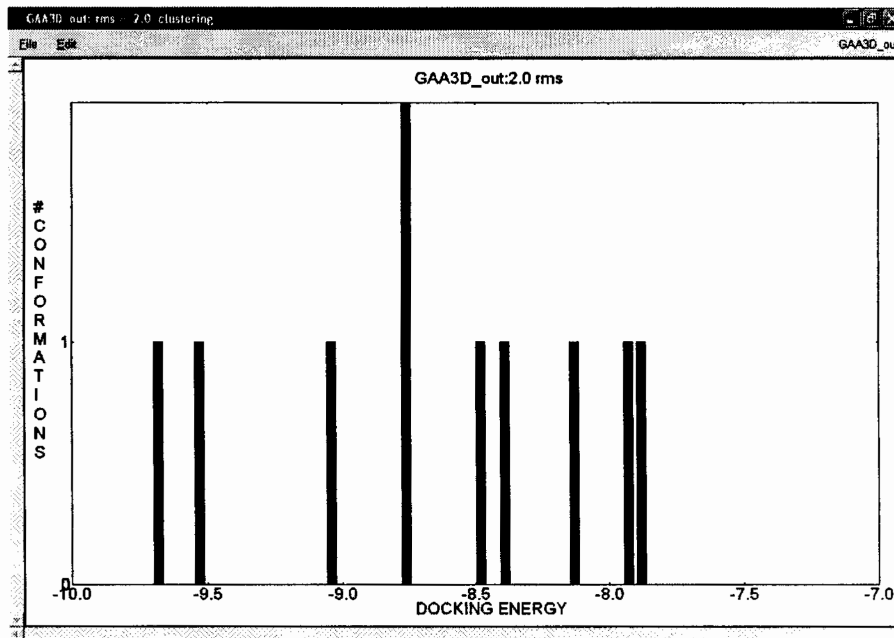


Figure 4.15: Clustering Histogram showing Docking Energy of all possible conformations with Ganoderic Acid A.

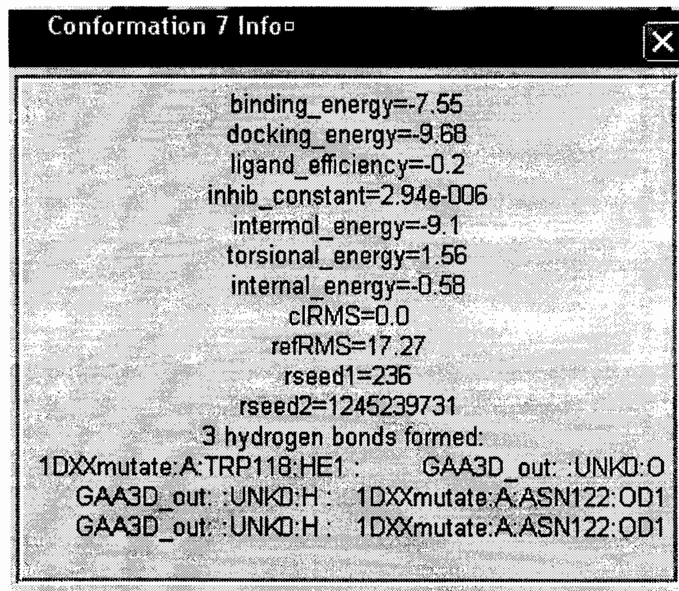


Figure 4.16: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Ganoderic Acid A and receptor.

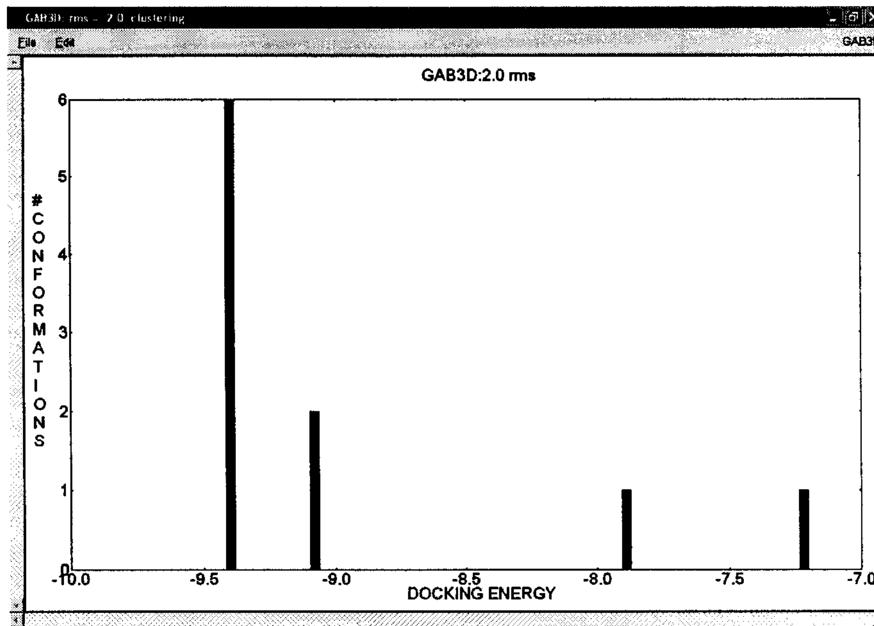


Figure 4.17: Clustering Histogram showing Docking Energy of all possible conformations with Ganoderic Acid B.

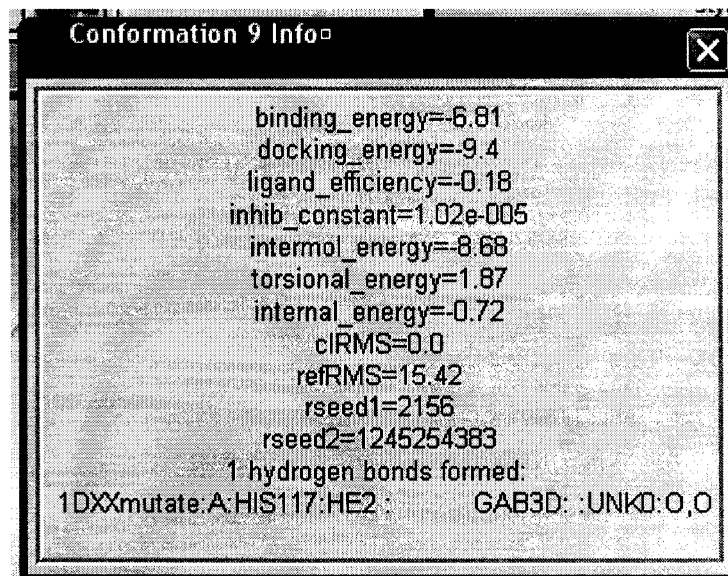


Figure 4.18: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Ganoderic Acid B and receptor.

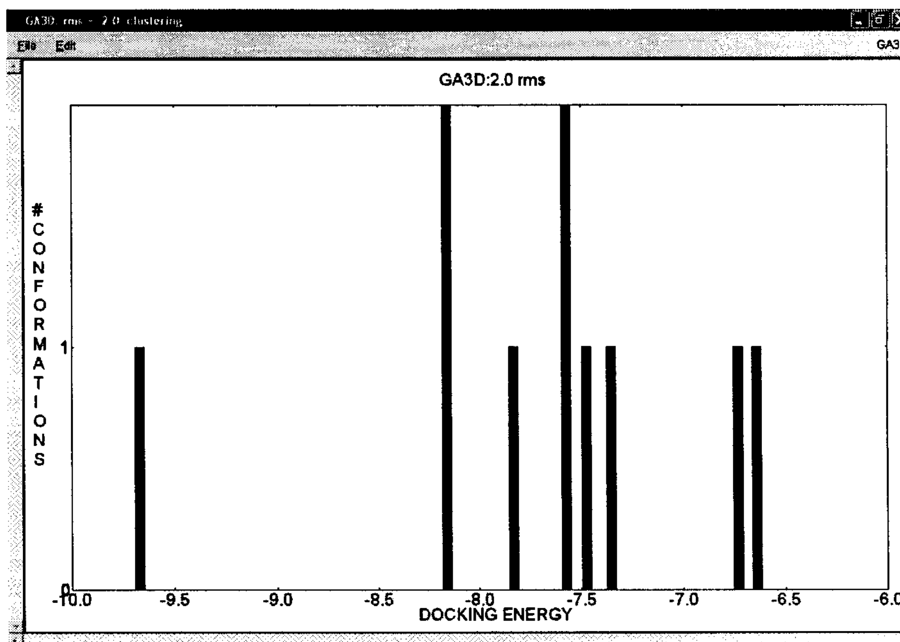


Figure 4.19: Clustering Histogram showing Docking Energy of all possible conformations with Ganoderol A.

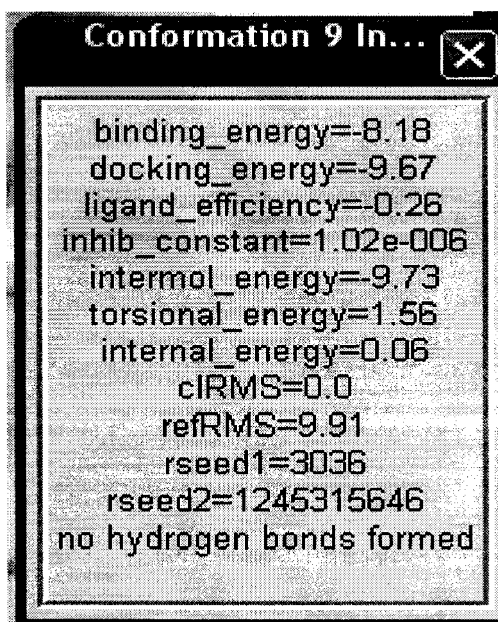


Figure 4.20: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Ganoderol A and receptor.

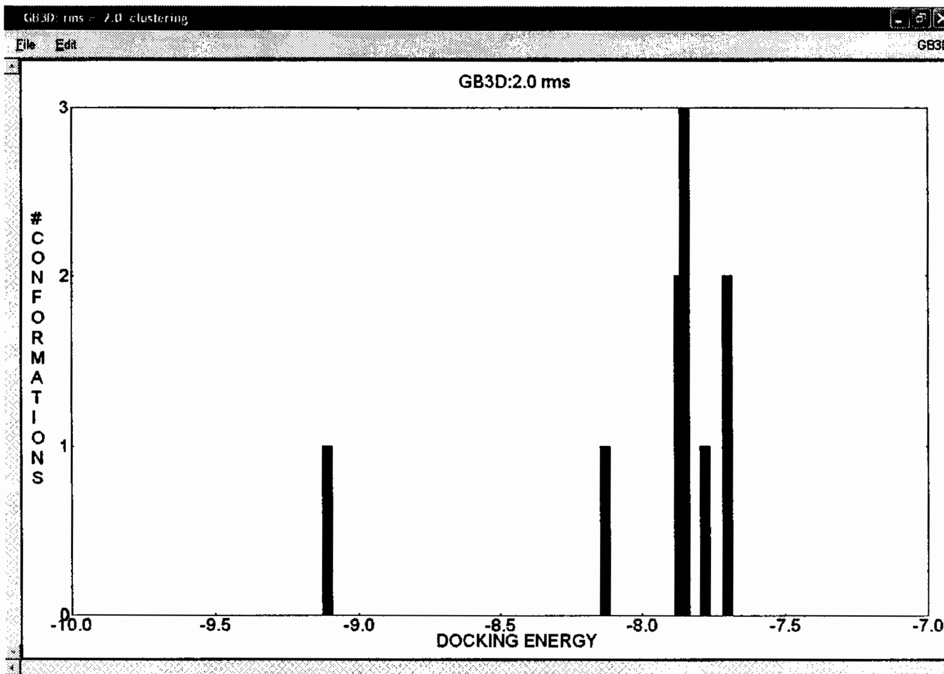


Figure 4.21: Clustering Histogram showing Docking Energy of all possible conformations with Ganoderol B.

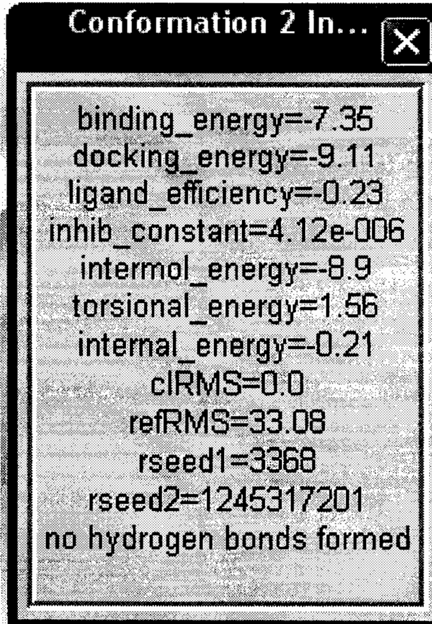


Figure 4.22: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Ganoderol B and receptor.

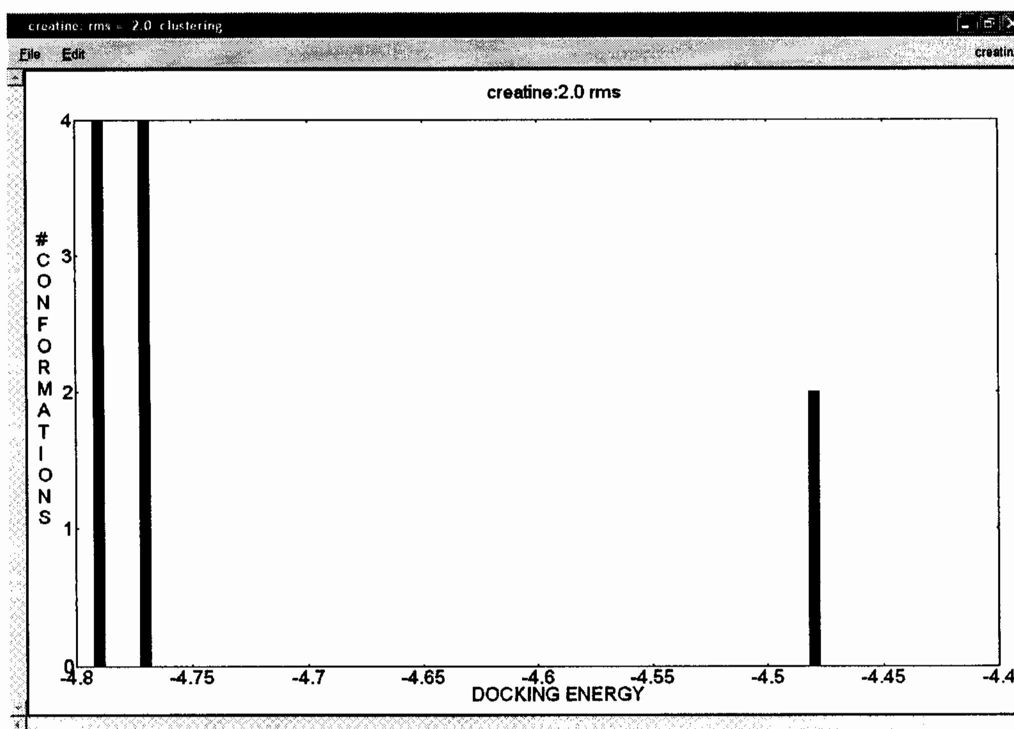


Figure 4.23: Clustering Histogram showing Docking Energy of all possible conformations with Creatine.

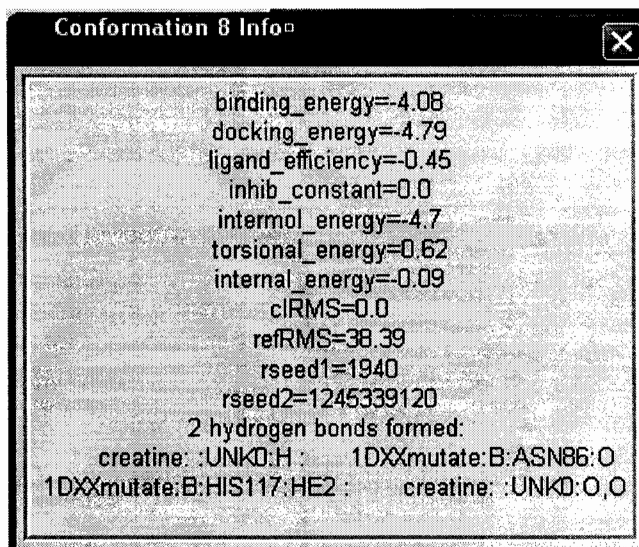


Figure 4.24: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Creatine and receptor.

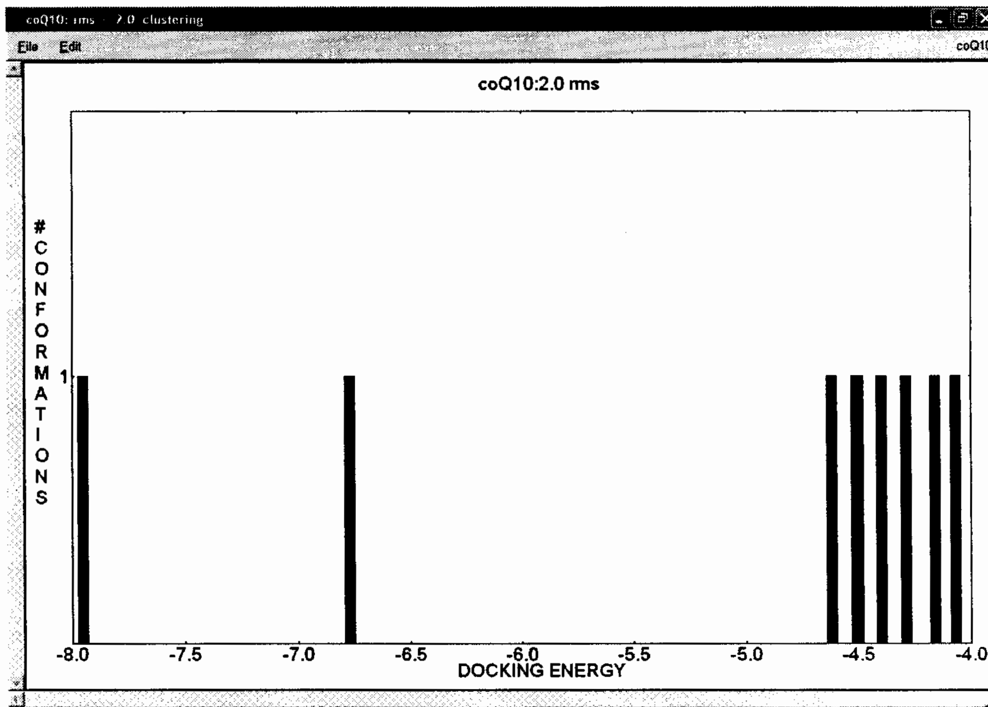


Figure 4.25: Clustering Histogram showing Docking Energy of all possible conformations with Coenzyme Q10.

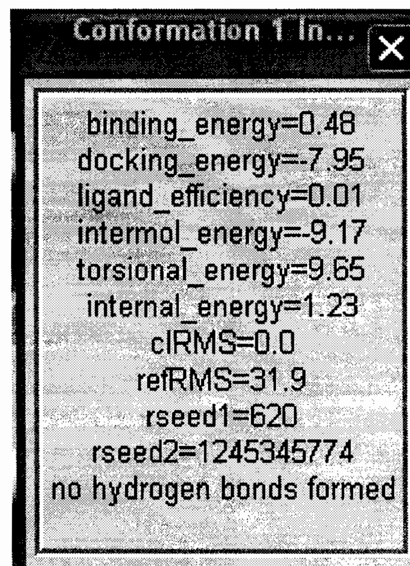


Figure 4.26: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Coenzyme Q10 and receptor.

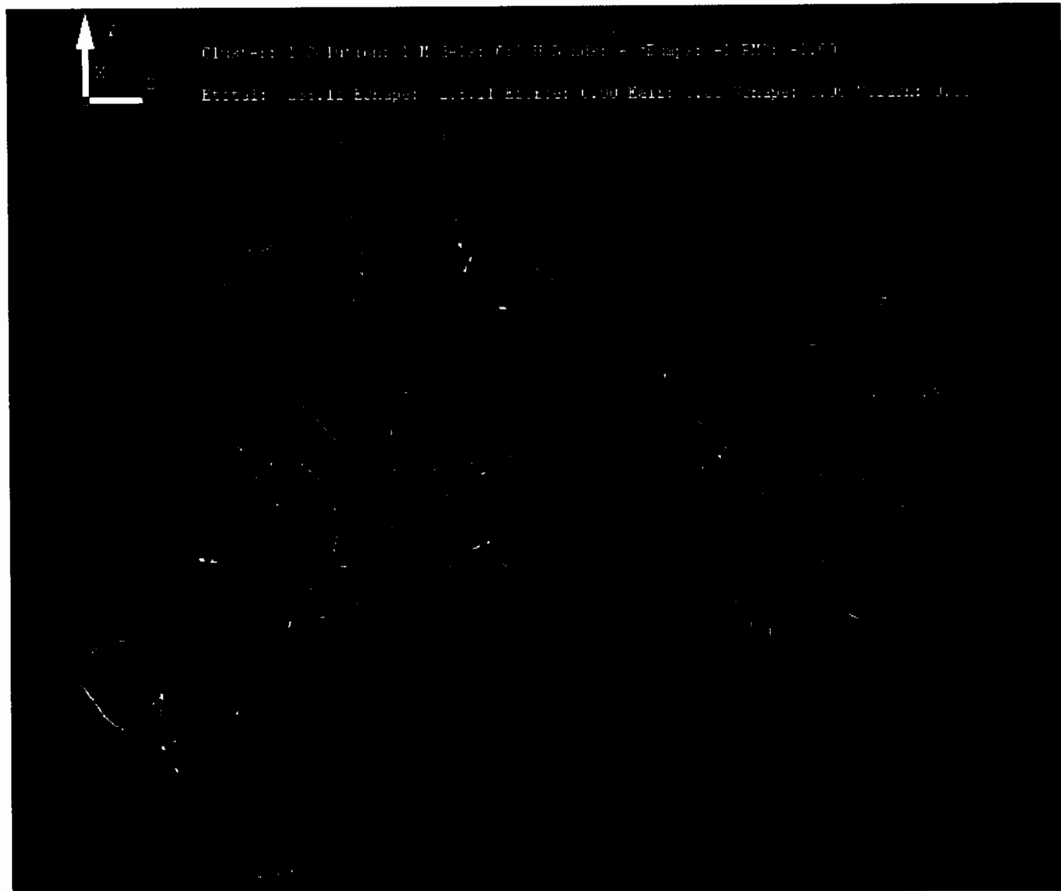


Figure 4.27: Dystrophin-Ganoderic Acid A complex showing Total Energy of Complex as -238.11.

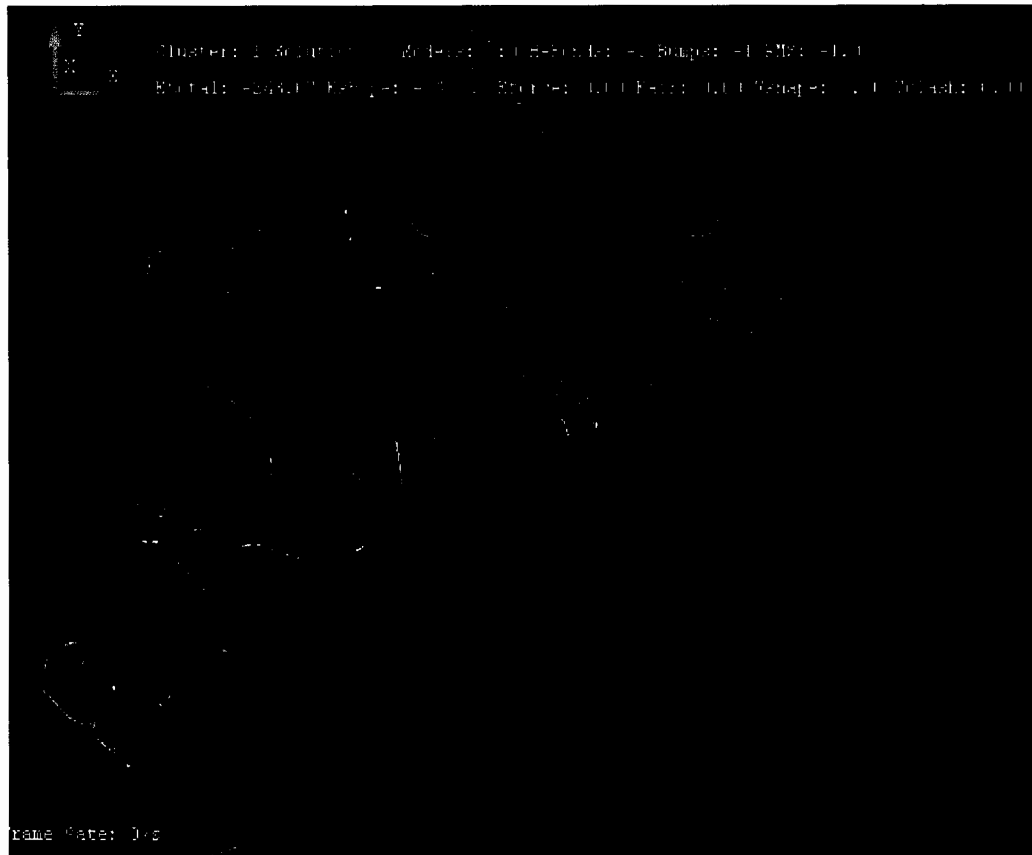


Figure 4.28: Dystrophin-Ganoderic Acid B complex showing Total Energy of Complex as -243.07.

Figure 3.2: An affected Individual (III-4) from family A with Syndactyly.

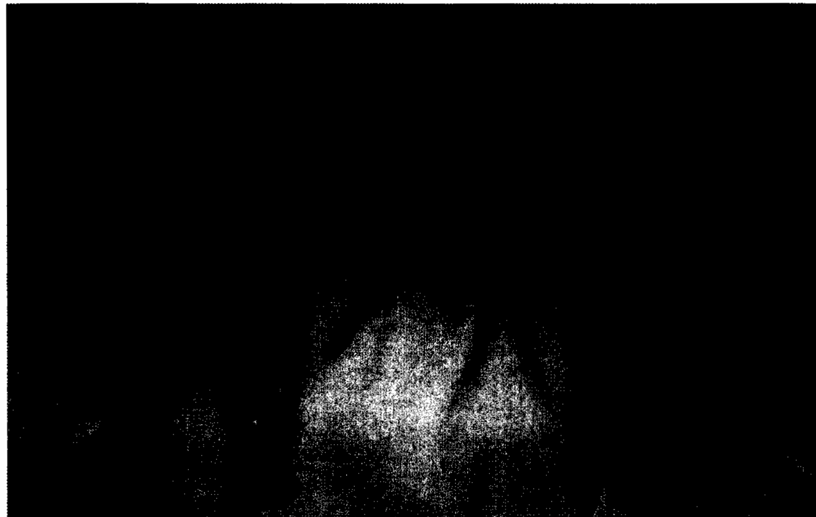


Figure 3.3: An affected Individual (III-4) from family A with Syndactyly.

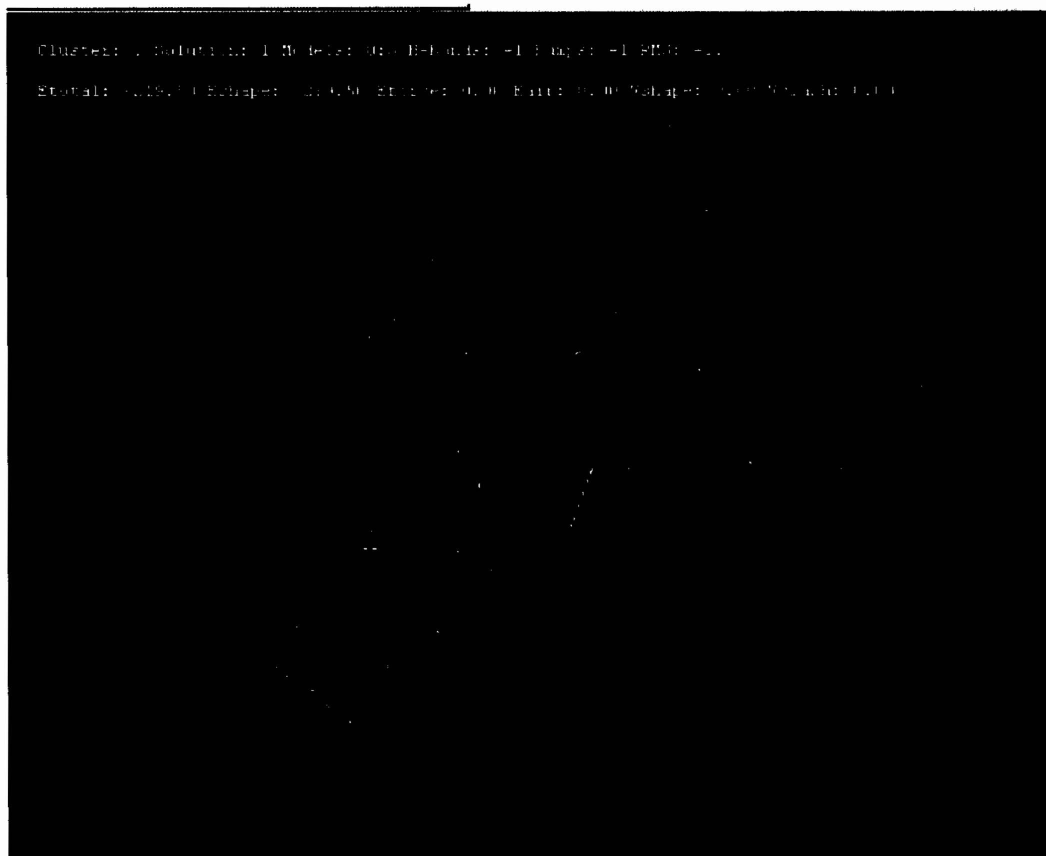


Figure 4.29: Dystrophin- Ganoderol A complex showing Total Energy of Complex as -219.50.

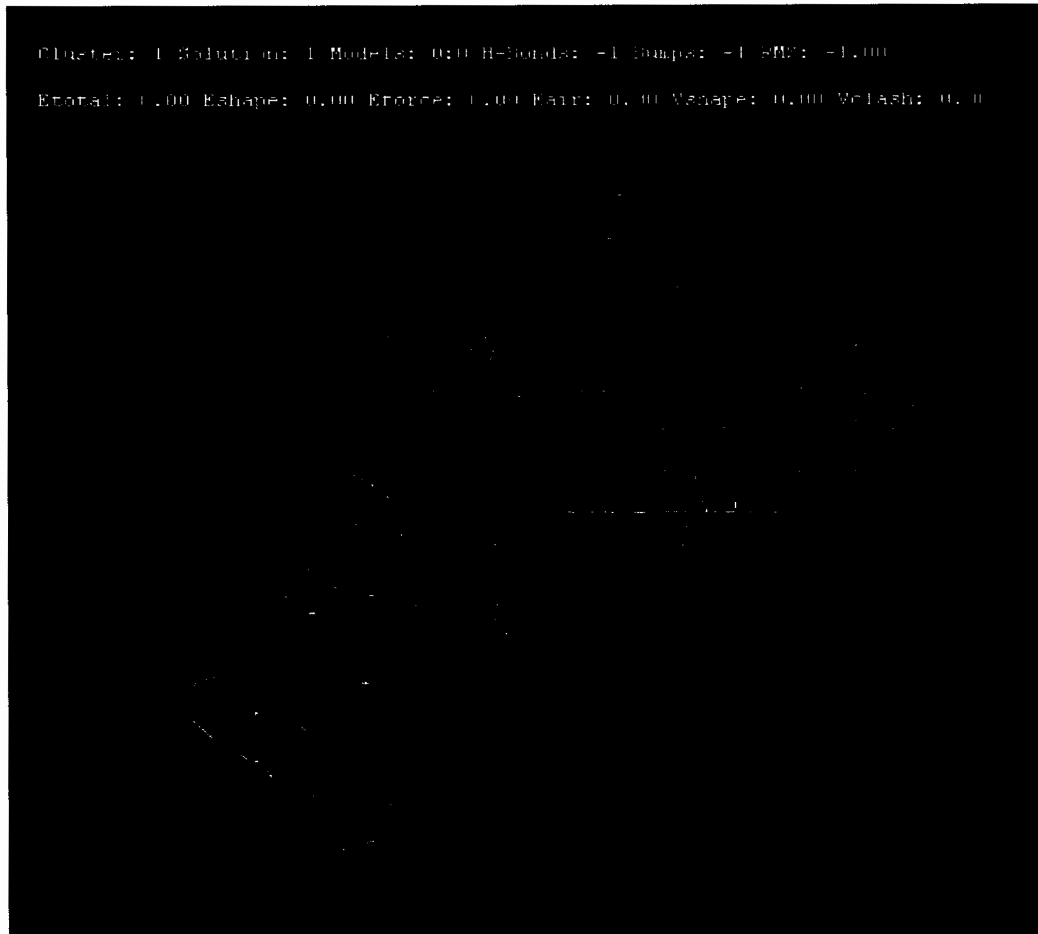


Figure 4.30: Dystrophin- Ganoderol B complex showing Total Energy of Complex as 0.00.

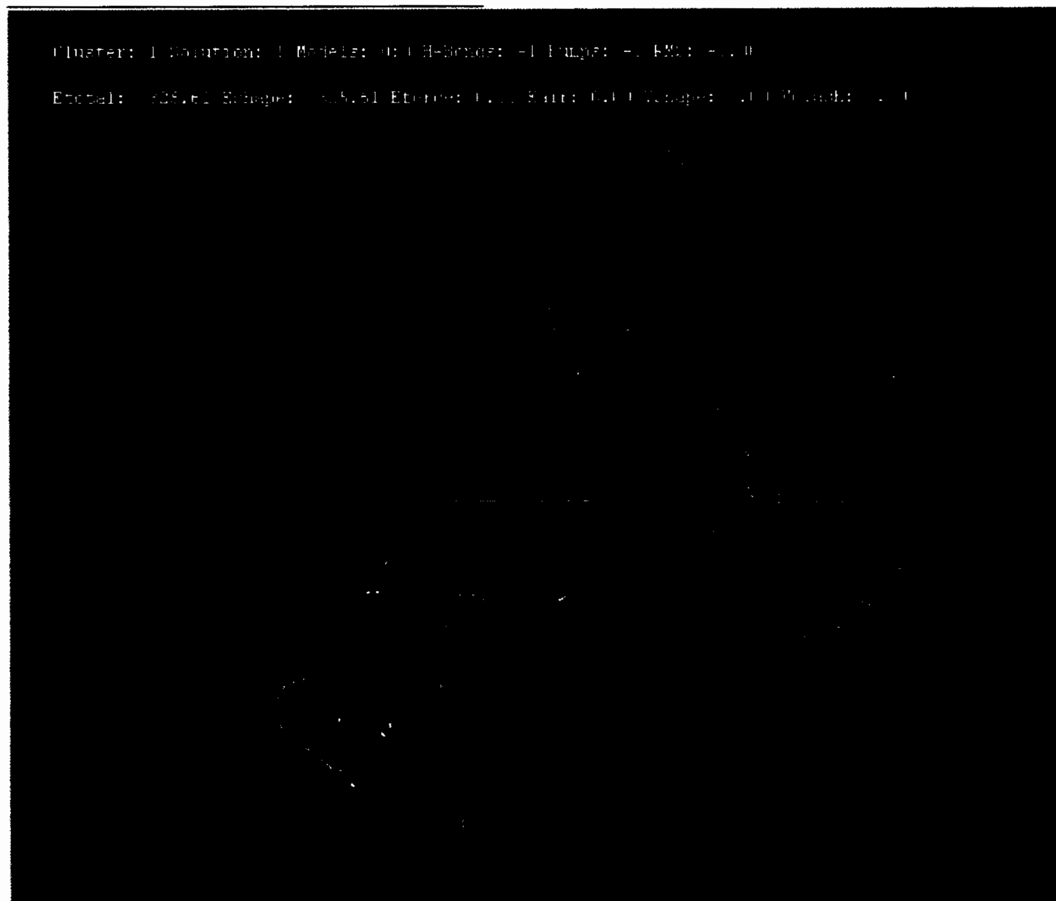


Figure 4.31: Dystrophin-CoenzymeQ10 complex showing Total Energy of Complex as -325.61.

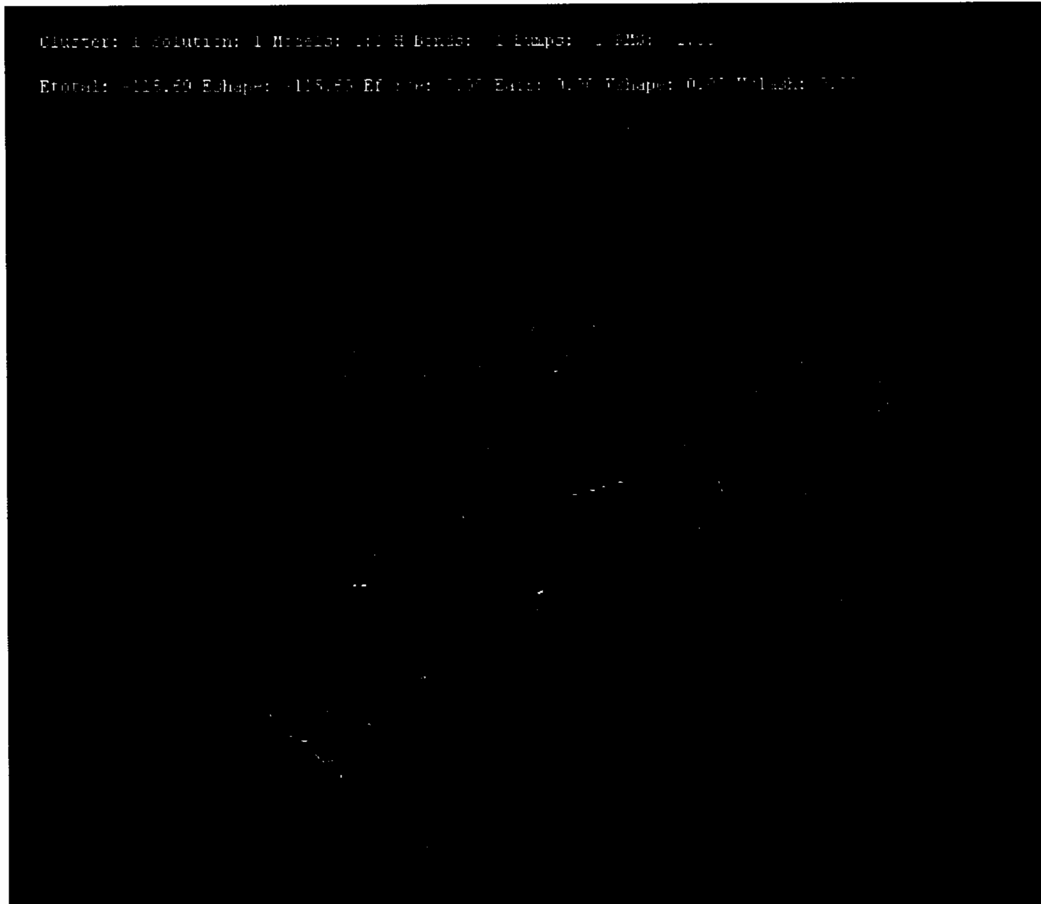


Figure 4.32: Dystrophin- Creatine complex showing Total Energy of Complex as -115.69.

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