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Biochemistry, Cytogenetics and *DMD* Gene Mutations in South Indian Patients with Duchenne Muscular Dystrophy

A. Meyyazhagan[1,2*], N. M. Raman[8], M. Easwaran[7], B. Balasubramanian[9], K. Alagamuthu[11], H. Kuchi Bhotla[2], S. Shanmugam[10], K. Inbaraj[4], M. Ramesh Kumar[6], P. Kumar[7], L. Thangamani[7], S. Piramanayagam[7], V. Anand[5], Y. Mohd[5], S. Park[9], O. Teijido[1,2], J.C. Carril[1,2], P. Cacabelos[1], S. Keshavarao[2] and R. Cacabelos[1,2*]

[1]EuroEspes Biomedical Research Center, Institute of Medical Science and Genomic Medicine, Corunna, Spain
[2]Genomic Medicine, Continental University Medical School, Huancayo, Peru
[3]Human Genetics Laboratory, Department of Zoology, School of Life Sciences, Bharathiar University, Bharathiar 641 046, Tamil Nadu, India
[4]Department of Conservation Biology, Bharathiar University, Bharathiar 641046, Tamil Nadu, India
[5]Medical Genetics and Epigenetics Laboratory, Department of Human Genetics and Molecular Biology, School of Life Sciences, Bharathiar University, Bharathiar 641 046, Tamil Nadu, India
[6]Department of surgery, KMCH Hospital, Coimbatore, Tamil Nadu, India
[7]Department of Bioinformatics, Computational Biology Laboratory, Bharathiar University, Bharathiar 641 046, Tamil Nadu, India
[8]Department of Biotechnology, Dr. G. R. Damodaran College of Arts and Science, Coimbatore, Tamil Nadu, India
[9]Department of Food Science and Biotechnology, College of Life Science, Sejong University, 209 Neundong-ro, Gwangjin-gu, Seoul-05006, South Korea
[10]Laboratory of Muscle Biology and Meat Science, Department of Animal Science, Chonbuk National University, 664-14 Duckjin-dong 1Ga, Jeonju City, Jeonbuk 561-756, South Korea
[11]College of Life Science, Nanjing Normal University, No.1 Wenyuan Road, Qixia District, Nanjing, Jiangsu Province-210023, China

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ABSTRACT Thirty children aged 3-10 years with clinically confirmed or suspected Duchenne Muscular Dystrophy (DMD) were analyzed for chromosomal aberrations using cytological preparations, biochemical changes using enzyme kit protocol, and deletions in the 26 exons of the *DMD* gene by targeting the mutations at the proximal and distal 'hot spot' regions of the dystrophin gene in South Indian patients with DMD. The frequency of chromosomal aberrations (both chromosomal and chromatid-type) and serum enzyme levels were significantly elevated in DMD subjects as compared to controls. Multiplex PCR assays revealed 27 patients having deletions in the *DMD* gene located at the distal 'hot spot' region. This study suggests that disease progression is directly associated with higher incidence of the deletions at the distal 'hot spot' of the *DMD* gene.

INTRODUCTION

Duchenne muscular dystrophy (DMD) afflicts 1 in 3500 live male births and is considered the most frequent fatal neuro-muscular genetic disease among children. This degenerative condition causes the deficiency or complete absence of dystrophin in striated muscles (Johnstone et al. 2017). Most affected mutations disrupt the open reading frame of the dystrophin gene, either due to spontaneous deletion or inheritance of the deleted gene, resulting in unstable truncated products. Despite the well-known symptoms associated with DMD, the diagnosis of DMD remains challenging in pediatric units or pediatric neurology units (Hendriksen et al. 2016).

*Address for correspondence:
Ramon Cacabelos and Meyyazhagan Arun
EuroEspes Biomedical Research Centre,
Institute of Medical Science and Genomic Medicine,
15165, Bergondo, Corunna, Spain
Phone: +34-981-780505,
Fax: +34-981-780511,
E-mail: rcacabelos@euroespes.com,
cytogenetics@ euroespes.com

Therefore, the present study is undertaken to analyse the serum enzymes of the identified subjects along with a genetic characterization of the *DMD* gene.

Among serum enzymes, creatinine kinase (CK) is a specific marker for muscle disease (El-Bohy and Wong 2005) whereas alanine aminotransferase (ALT), aspartate aminotransferase (AST), aldolase (A), and lactic dehydrogenase (LDH) are indicators of liver function. In children, serum levels of these enzymes associated with liver function are frequently tested even more than CK. Upon muscular dystrophy conditions, the biochemical assessment of these enzymes shows high levels since birth; therefore, analysis of enzyme activity levels in neonates allows the early diagnosis of the disease (Hassan et al. 2008; Nadkarni et al. 2008).

The *DMD* gene is structurally complex, with 79 exons and 8 promoters, comprising a total of 2.4 million base pairs, which makes it one of the largest genes known up to date (Ahn and Kunkel 1993). Mutations in this large gene generally result in a disturbance of the open reading frame during DMD protein production that either leads to the synthesis of a truncated, degraded protein or to a complete absence of the DMD protein (Aartsma-Rus et al. 2006). Approximately, sixty-five percent of the dystrophin mutations in the *DMD* gene are intragenic deletions; 25-30 percent account for point mutations, insertion and nucleotide changes (Nussbaum et al. 2001), and 5-10 percent are duplications that appear to be evenly distributed (Muntoni et al. 2003). The deletions are mostly clustered within the two known 'hot spot' regions; towards the 5' end, known as proximal 'hot spot' (Blake et al. 2002), and near the central part of the gene known as the distal 'hot spot'. As documented by Beggs et al. (1990), the clusters of these two hot spots represent the basis for the use of the multiplex PCR technique that allows the simultaneous screening of 19-26 exons and the detection of ninety-eight percent deletions. The development of rapid multiplex PCR assays for *DMD* deletion provided a means to analyse up to 10 exons in a single PCR reaction mix.

Objectives

The aim of this study was to perform a cytogenetic analysis using high-resolution G-banding techniques, biochemical analysis, and to perform a complete characterization of the *DMD* gene mutations leading to DMD in South Indian patients which will contribute to the early diagnosis of the disease.

MATERIALS AND METHODS

Selection of Subjects

A total of 60 subjects ($n_{Controls}$ = 30; n_{DMD} = 30) were selected for this study. The average age of 6.43±0.33 years and 7.5±0.44 years was observed among the DMD-affected and controls, respectively. Gender based differentiation reveals that 25 were male in both the test subjects and controls, which was selected among the population of Coimbatore, Tamil Nadu, India. All the subjects were recruited between three to ten years of age. Informed written consent was obtained from their parents and an open questionnaire was directed towards the patients and controls to acquire relevant details on age, life style factors and medical history. All controls belonged to the same ethnic origin as the recruited patients. The study followed the ethical procedures and informed consent was obtained from the subjects. Blood samples were collected from the subjects and analysed for biochemical analysis, chromosomal aberrations (CA) and *DMD* gene mutation screening. The work was carried out in accordance with the ethical standards of the 1964 Declaration of Helsinki (WMA General Assembly 2001).

Cytogenetic Study

All chemical reagents were purchased from Sigma Chemical (St. Louis, MO), except for colcemid, which was obtained from Gibco Laboratory (Grand Island, NY). Blood samples were set up to establish cell cultures according to the standard procedures of our laboratory (Moorhead et al. 1960). Briefly, 0.5 mL whole blood was added to 4.5 mL RPMI 1640 medium supplemented with ten percent fetal bovine serum, 2 mM L-glutamine, one percent streptomycin–penicillin antibiotics and 0.2 mL reagent-grade phytohemagglutinin, and incubated at 37°C. After 71 hrs, the cultures were treated with 0.1 µg/mL colcemid to block cells in mitosis. The lymphocytes were harvested at 72h by centrifuging cells to remove culture medium (800-1,000 rpm, 7 min) to which hypotonic solution (KCl 0.075 M) was

added at room temperature and incubated for 20 min to swell the cells. The cells were treated twice with fixative (methanol and acetic acid [3:1 vol/vol]). Cytological preparations were made by placing two to three drops of the concentrated cell suspension onto slides wetted with ice-cold acetic acid (60%) and were carefully dried on a hot plate (56°C for 2 min). For Chromosomal Aberration (CA) analysis, 100 complete metaphase cells of the first cell cycle were evaluated under a microscope (x100) to identify numerical and structural CA according to the International System for Human Cytogenetic Nomenclature (ISCN 1995). Data were registered on master tables and later transferred to a computer file.

Biochemical Analysis

Serum enzyme (CK, LDH, A, AST, ALT) analysis was performed based on Enzyme kit protocol (kits supplied by Sigma®).

Genomic DNA Isolation and Multiplex PCR

DNA was isolated from three to four ml of whole blood was collected in a sterile purple top tube containing EDTA as an anticoagulant factor. Whole genomic DNA was collected by following the instructions from the Bangalore Genei-Frozen blood DNA extraction kit. *DMD* gene deletions were detected using four multiplex PCR reactions. A total of 26 exons were analysed, six with multiplex set-1, seven with multiplex set-2, seven with multiplex set-3, and six with multiplex set-4, as previously reported by Chaudhary et al. (2008) and Chaudhary 2009. The reaction mixture was made in a volume of 50 µl containing 1X PCR buffer, 10 mM dNTPs, 1M $MgCl_2$, 200 ng/ml of each primer, 5000 U/ml Taq DNA polymerase and 2µl of 200 ng/ml genomic DNA of each sample. The amplification was carried out as a 'hot start' followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 70°C for 2 min. Cycling was concluded with a final extension at 70°C for 5 min. The PCR products were run by electrophoresis in a two percent agarose gel and visualised under UV after ethidium bromide staining.

Statistical Analysis

All statistical analyses were performed using the software SPSS (version 17) to assess the group statistics for subjects and controls as mean±SD. For statistical data inference, the t test for independent variables and ANOVA were used to compare mean values of the quantitative variables. Significance was considered at p value below 0.05.

RESULTS

The present study focused in the distribution spectrum of mutations in the proximal and distal 'hot spot' regions of the DMD gene together with the screening of cytogenetic aberrations and biochemical alterations in South Indian patients.

Serum enzyme (CK, LDH, A, AST, ALT) level of DMD patients were 1,358.66±94.52 IU/L, 560.67±393.07 IU/L, 14.97±0.35 U/L, 262.3±1.07 U/L, and 554.37±10.13 U/L, respectively and 75.27±6.54 IU/L, 212.2±12.51 IU/L, 2.68±0.23 U/L, 0.37±9.12 U/L, 8.56±0.42 U/L, respectively, controls. Serum enzyme levels and the frequency of chromosomal aberrations were found to be significantly higher ($p<0.01$) in DMD cases (Fig. 1).

Figure 2 displays the total number of major and minor CAs seen in both DMD patients and controls. In DMD patients the major CAs were found higher in chromosomes 1, 4, 9, 11, 12, 21 and X (Major CA, n= 21, and minor CA, n= 9). A lesser number of aberrations were observed in chromosomes 2, 5, 8, 13 and 18 in DMD. The major alterations observed were deletions and translocations mainly in the chromosome 9, 11, 12, and X. The mean of chromosomal aberrations (CSA and CTA) was significantly higher ($p<0.05$) in DMD patients (1.87±0.16, 0.73±0.115) than that in healthy controls (0.17±0.07, 0.37±0.09).

Exon deletions were observed in 27 out of 30 patient samples, where the remaining three samples did not show any deletions for all of the 4 multiplex sets tested (Table 1). The deletions were confined to the distal 'hot spot' region of the DMD gene that included exons 45 to 53. The number of exons deleted in a given patient varied from three in patients 4, 6, 9, 11, 14, 15 and 22, to at least nine exon deletions observed in patients 1, 2, 10, 17 and 25. Thirteen patients had died by the time when current clinical manifestations were recorded and each of them had 8 to 9 exon deletions. No deletions were detected in the proximal 'hot spot' region of the DMD gene in 30 patients which included exons 1-19.

Interestingly, the analysis between the CK levels and the number of deletions among the DMD subjects reveal a positive correlation between them (Fig. 3). Increase of CK levels indicates an increase in the same subjects having more than four duplications. This correlation validates the requirement to analyze the genetic profile of the subject of interest along with their biochemical testing.

DISCUSSION

The current assessments among DMD population include invasive procedures such as bi-

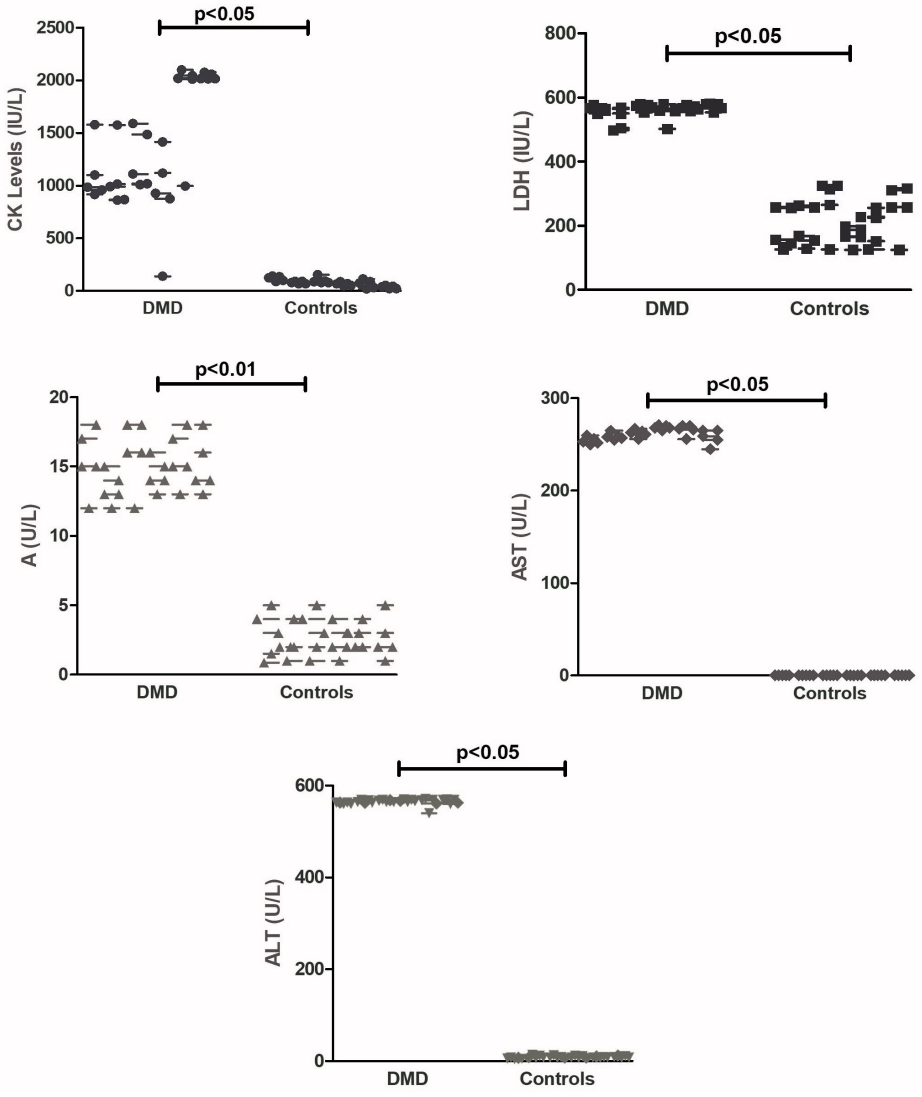

Fig.1. Enzymatic activity in patients with DMD vs Controls.
CK: Creatine kinase; LDC: Lactate dehydrogenase; A: Aldolase; AST: Aspartate aminotranseferase; ALT: Alanine aminotransferase. The values are expressed as Mean± SD; Value significant at p<0.01 and p<0.05 level (DMD vs Control subjects) have been identified.

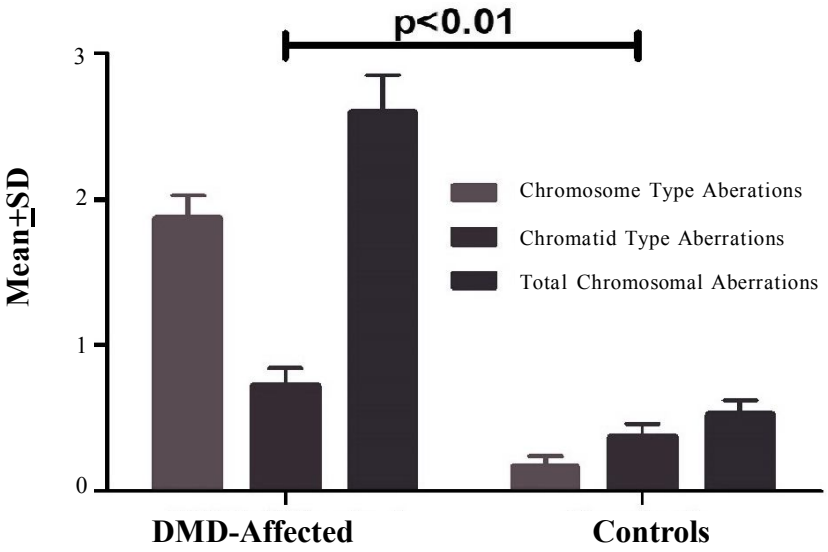

Fig. 2. Frequency of chromosomal aberrations in DMD and control subjects. The values are expressed as Mean ± SD; significant values at p<0.01 level (DMD-Affected subjects vs Control subjects) have been identified

opsies and complex physical tests which are highly unsuitable among children. Objective outcome measures which are less invasive will help in evaluation of the disease progression, drug development and clinical trials (Hathout et al. 2016).

Although the analysis of serum CK levels alone has been termed as sensitive and simple, it has been widely observed that the economic restrictions pose a negative influence the clinical analysis of obvious incidents of DMD in children (Zhu et al. 2015). Globally it has been accepted that the analysis of transaminases along with the CK levels should be evaluated with equal importance for predicting and concluding the conditions of muscular dystrophy and DMD in particular (Hoogerwaard et al. 2005).

Similar to previous reports by Zhu et al. (2015), the present study also shows abnormally high levels of serum enzymes among DMD subjects when compared to controls. The use of serum levels as an index for characterising the type of muscular dystrophy has also been undertaken by Zhang et al. (2012) who dealt with DMD, Becker's muscular dystrophy, facioscapulohumeral dystrophy, limb girdle muscular dystrophy and Emery-Dreifuss muscular dystrophy. The mechanism by which these serum levels become abnormal in all forms of muscular dystrophy is still not conclusive. CK level is an important laboratory parameter because DMD positive patients have more frequent dilated cardiomyopathy and abnormal gait (Sheffali et al. 2005). Elevations in AST have been associated with hepatocellular damage associated with cardiac, brain, renal and blood disturbances, whereas ALT abnormalities lead to cellular damage in skeletal muscles (Pratt and Kaplan 2000). It is assumed that the release of CK under pathological conditions such as muscular dystrophy occurs along with the leakage of transaminases from muscle membrane (Zhu et al. 2015). However, the fold increase in the observations of serum enzymes may vary due to different methodologies in the detection of serum enzymes or conditions involved in the collection of blood samples. The fold increases are much higher in the present study than the ones reported by Zhu et al. (2015) using the clinical data obtained from Chinese patients.

The conventional cytogenetic analysis of chromosomal aberration (CA) frequencies in peripheral blood lymphocyte has been internationally standardized; furthermore, contrary to alternative measures, this biomarker has been validated in terms of its association with subsequent disease outcomes. It is generally accept-

Table 1: Distribution of deletions in 30 DMD-affected patients

Patient sample	*Exons deleted*	*No. of deletions*	*Clinical status*
*DMD 1	45, 46, 47, 48, 49, 50, 51, 52, 53	9	Asymptomatic
DMD2	45, 46, 47, 48, 49, 50, 51, 52, 53	9	Moderate
DMD 3	46, 47, 48, 49, 50, 51, 52, 53	8	Asymptomatic
**DMD 4	51, 52, 53	3	Moderate
DMD 5	No deletions found in both distal and proximal regions of DMD gene	0	Mild
DMD 6	51, 52, 53	3	Severe
**DMD 7	45, 46, 50, 51, 52, 53	6	WCB
DMD 8	47, 48, 49, 50, 51, 52	6	Moderate
DMD 9	48, 49, 50	3	Mild
DMD10	45, 46, 47, 48, 49, 50, 51, 52, 53	9	Asymptomatic
*DMD11	46, 49, 51, 53	3	Severe
*DMD12	47, 48, 49, 50, 51, 52	6	Moderate
DMD13	46, 47, 48, 49, 50, 51, 52, 53	8	Severe
DMD14	51, 52, 53	3	Severe
DMD15	49, 50, 51	3	WCB
**DMD16	46, 49, 51, 53	4	Severe
DMD17	45, 46, 47, 48, 49, 50, 51, 52, 53	9	Mild
*DMD18	45, 46, 47, 48, 49, 50, 51, 52	8	Moderate
DMD19	46, 47, 48, 49, 50, 51, 52, 53	8	Mild
DMD20	46, 49, 51, 53	4	Mild
**DMD21	No deletions found in both distal and proximal regions of DMD gene	0	WCB
*DMD22	51, 52, 53	3	Dead
DMD23	46, 49, 51, 53	4	Asymptomatic
DMD24	46, 47, 48, 49, 50, 51, 52, 53	8	Moderate
DMD25	45, 46, 47, 48, 49, 50, 51, 52, 53	9	Asymptomatic
DMD26	46, 49, 51, 53	4	Mild
DMD27	46, 47, 48, 49, 50, 51, 52, 53	8	Severe
DMD28	No deletions found in both distal and proximal regions of DMD gene	0	Mild
**DMD29	46, 47, 48, 49, 50, 51, 52,53	8	Asymptomatic
**DMD30	46, 47, 48, 49, 50, 51, 52, 53	8	Moderate

Mild - fatigue and/or any detectable weakness including clumsiness, falling, abnormal gait, toe walking and slow running in the absence of positive Gower's signs; Moderate - positive Gower's sign, difficulty with stairs and/or waddling gait; Severe - inability to rise without assistance and/or walking only with effort and/or severe wasting of muscle; WCB - wheelchair bound; **Dilated cardiomyopathy; *With an affected brother

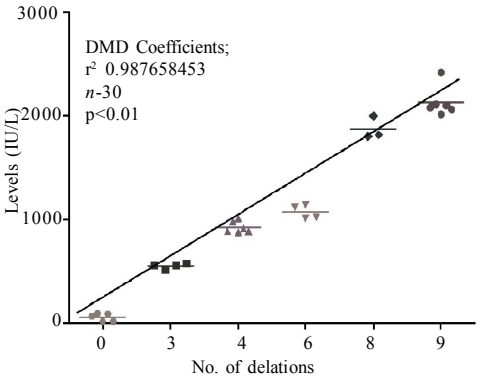

Fig. 3. Correlation between CK levels and duplications in DMD patients. The values are expressed as coefficients; values have been found significant at $p<0.01$ with $r^2= 0.98$.

ed that an increased frequency of CA in PBL indicates a clastogenic exposure and hence related to cancer at a population level (Bonassi et al. 2004; Hagmar et al. 2004; Rossner et al. 2005).

Botstein et al. (1980) published the first study of *DMD* gene by providing the theoretical basis for the use of restriction fragment length polymorphisms (RFLPs) to find genes for diseases of the unknown gene product. Murray et al. (1982) described the first DNA marker for the *DMD* genes and with the important additional attribute of the sequences flanking the *DMD* locus (Davies et al. 1983) the interest for *DMD* was further equated. This rapid progress helped the identification of DMD in girls with chromosome translocations that involved Xp21 (Brunet et al. 2009), by denoting the general area of

the *DMD* gene on the short arm of the X chromosome.

DMD and Becker muscular dystrophy are two allelic forms of an X-linked muscle disorder exhibiting phenotypic heterogeneity (Medori et al. 1989; Tuffery-Giraud et al. 2009). The phenotype of severe DMD has been associated with duplications or deletions that shift the reading frame whereas the mild condition of Becker muscular dystrophy maintains the reading frame despite of the presence of deletions or duplications (Lalic et al. 2005).

Molecular studies addressing mutation analysis of DMD gene in South Indian patients are very few (Singh et al. 1997; Mallikarjuna Rao et al. 2003) focusing mainly on establishing multiplex PCR methods for the detection of deletion mutations and their distribution in the 'hot spot' regions of the *DMD* gene. However, studies failed to diagnose causative deletions outside the 'hot spot' regions in three out of fifteen cases clinically confirmed as DMD (Shrimpton et al. 2001), subsequently highlighting the limitation of this approach and suggesting that it might be used as a screening rather than a confirmatory tool. Other more robust, fluorescent based multiple ligation probe assay (MLPA) should be considered as the method of choice both for confirming deletion mutations by scanning the 79 exons of the DMD gene (Lai et al. 2006), as well as testing for carrier status in unaffected females without X-autosome translocations (Nussbaum et al. 2001). The methodology used in this study was based on multiplex PCR targeting 26 exons.

In most studies, 80-91 percent of deletions occurred in the distal region of the *DMD* gene (Singh et al. 1997) and the deletion rates were 42-52 percent. (Odinokova et al. 1996). In the study of 160 Indian patients from all over the country, the deletion rate of 64.4 percent and 69.7 per cent was in the distal hot spot region. This study did not find any ethnic differences in the deletion patterns of the *DMD* gene (Banerjee and Verma 1997). In a study based on the Eastern India population, the deletion rate of sixty-three percent and seventy-nine percent was at the distal hot spot region (Basak et al. 2006).

Singh et al. (1997) reported a deletion rate of seventy-three percent in a Northern Indian population which included both DMD and Becker muscular dystrophy. The reported deletion rate of 62.1 percent and seventy-eight percent was located at the distal hot spot region in a Southern Indian DMD population (Mallikarjuna Rao et al. 2003). The authors of this study concluded that the lower deletion rate in their population, when compared to the North Indian population, may be related to the ethnic differences in the two populations. The deletion rate reported among 25 Western Indian DMD patients of (72%) was mostly located at the 3' hot spot region (Khalap et al. 1997). The deletion rate found in our study was about seventy percent and was similar to the frequency reported from the other parts of India (Nadkarni et al. 2008). The reported frequency of *DMD* gene deletions in other Asian countries is quite variable: 40.7 percent in Pakistan (Hassan et al. 2008), 66.25 percent in China (Wang et al. 2008), and 61.1 percent in Egypt (El Sherif et al. 2007).

Patients with fewer deletion mutations suffer mild to moderate symptoms suggestive of DMD. Deletions are used as predictive markers among brothers of the subjects and first-degree male relatives of the affected sibling. Furthermore, these deletions can be used as probands in prenatal diagnosis in subsequent pregnancies (Kumari et al. 2003).

Although CK levels coincided with muscle wasting in our candidates, studies have shown *de novo* mutations in the *DMD* gene that may cause DMD to occur in families without prior disease history (Emery 2002; Hoogerwaard et al. 2005), where all the family members of the affected ones present normal serum CK levels. This means that the other brothers of the patient are not affected and even the mothers and sisters may not carry DMD mutations. It has been reported that seventy percent of the carrier females show slight elevations in serum CK levels (Hoogerwaard et al. 2005).

Serum CK concentration gradually decreases with aging as a result of the progressive elimination of dystrophic muscle fibers that are the source of the elevated serum CK concentration (Hoffman et al. 1988). The correlation between the CK levels and duplications observed, for the first time, in this study which coincides with Sumita et al. (1998) who compared the concentrations of CK levels in carriers younger than age 20 years with those older than age 20 years.

CONCLUSION

Our study attempts to understand the correlation between the levels of serum enzymes and

genetics among South Indian DMD patients. The implication of this neurodegenerative condition has been observed among the family members of the patients in a psychologically strenuous fashion. This progress in genetic technology with the analysis of frequency of deletion mutations in the DMD gene poses as a helpful guide in diagnosis.

RECOMMENDATIONS

According to the present results, the researchers of this study recommend to use the non-invasive strategy of analyzing the serum enzymes along with the genetic analysis to detect DMD in pediatrics.

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