ORIGINAL ARTICLE
COMPARISON OF SERUM CREATINE KINASE ESTIMATION WITH SHORT TANDEM REPEATS BASED LINKAGE ANALYSIS IN CARRIERS AND AFFECTED CHILDREN OF DUCHENNE MUSCULAR DYSTROPHY

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Background: Duchenne Muscular Dystrophy (DMD) is an X-linked recessive lethal, genetic disorder characterised by progressive weakness of skeletal muscles which is untreatable and transmitted to males by carrier females. Advances in laboratory techniques now focus direct mutational analysis as the most reliable and indirect analysis based on Short Tandem Repeats (STR) based linkage analysis as feasible, inexpensive, and efficient method for carrier detection and prenatal diagnosis. The objective of this study was to compare the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficiency of Serum Creatine Kinase (SCK) with Short Tandem Repeats (STR) based linkage analysis in carriers and affected children of Duchenne Muscular Dystrophy. **Methods:** The study was carried out from Dec 2006 to Dec 2007 in families having index clinical cases of DMD who were referred from different hospitals for evaluation/workup of DMD. SCK was done as a preliminary investigation in all index cases. The PCR assay with STR based linkage analysis with Intron 44, 45, 49 and 50 of DMD gene were performed in all families. Six families were informative with Intron 44 of DMD gene and one family was non-informative with all four intronic markers of DMD. SCK analyses were done in all the family members and compared with PCR analysis in informative families. SCK was not performed on Chorionic villous sample (CVS) done for prenatal diagnosis of DMD, and CVS and non-informative family members were excluded from the study. **Results:** In carriers of DMD, the sensitivity and negative predictive value of SCK were 33.3%, and specificity and positive predictive were 100% with diagnostic efficiency of 50%. In affected cases of DMD the sensitivity and negative predictive value of SCK were 100%, and specificity and positive predictive were 91% and 88.8% respectively and diagnostic efficiency of 94.1%. **Conclusion:** The SCK is an excellent screening test for affected cases of DMD. For carrier identification we have to resort on PCR analysis so as to provide safer diagnostic tool for genetic counselling and prenatal diagnosis.
Keywords: Duchenne muscular dystrophy, Creatine Kinase, Carrier detection, Short Tandem Repeats

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder characterised by progressive skeletal muscle weakness.[1] It is also called pseudo hypertrophic muscular dystrophy.[2] Early gross motor mile stones may be delayed and a delay in walking until 12 months or more is frequent. The child may be slow to walk, falls frequently, and has difficulty in getting up, running, jumping and climbing stairs.[3] The disease presents as early as 2–3 years of age with delayed motor milestones.[4] An important early sign is an inability to rise from the floor or a chair without pressing on the thighs (Gower's sign).[5] Due to X-linked recessive inheritance the disease is most often transmitted to male offspring by asymptomatic female carriers. Cytogenetic and molecular studies have localised the DMD locus on Xp21 (the short arm of the X chromosome band 21).[6] Children of a carrier female have a 50% chance of inheriting the mutation, the sons who inherit the DMD mutation will be affected with the disorder, while daughters who inherit the mutation will be carriers. In rare circumstances females can also present with this disease, which can be explained on the basis of the Lyon hypothesis.[7] Therefore best preventive strategy against this incurable and lethal disease is carrier detection in DMD families.

The best non-invasive diagnostic modality for DMD carriers is PCR analysis either by mutation detection or linkage analysis.[8] As mutation analysis is expensive and requires sophisticated laboratory instrumentation, so the indirect approach of linkage analysis by using Short Tandem Repeats (STR) in the introns of dystrophin gene is alternative, reliable and feasible technique for carrier detection in DMD families.[9] Unfortunately most of the laboratories in our country are not equipped with molecular diagnostic facilities, so we have to still use biochemical methods for screening of affected individuals and carriers of disease.

Traditionally the Serum Creatine Kinase (SCK) is the most reliable, simple and reproducible biochemical marker for DMD patients[10], but has limited role in detection of female carriers[2]. Newborn screening for DMD based on CK analysis was introduced in Wales in 1990 by implementing a strict protocol.[11] Other biochemical markers have been used to study DMD[12] but the enzymes analysis cannot

be used solely and confidently for carrier detection and prenatal diagnosis[13].

This study was planned to determine the diagnostic efficiency of SCK by comparing it with linkage based STR analysis in carriers and affected children of DMD.

MATERIAL AND METHODS

It was a cross-sectional study carried out from Dec 2006 to Dec 2007 at the Department of Chemical Pathology and Endocrinology, after approval of ethical committee of AFIP, in collaboration with Department of Molecular Biology, Armed Forces Institute of Pathology Rawalpindi, including unrelated families having index cases of DMD. The index cases were the patient with history of early onset muscular dystrophy (at the age of two to three years) having characteristic clinical features of DMD and with positive Gower's sign. The sampling method was non-probability purposive sampling. Informed written consent was taken from parents of the index cases.

About 10 ml of blood was drawn; three ml of blood was put in Potassium Ethylene Diamine Tetra Acetic Acid (K-EDTA) bottle for Deoxynucleic Acid (DNA) extraction and PCR analysis and the remaining blood in two plain tubes for analysis of SCK. The non-informative family on PCR analysis was excluded from the study. For prenatal diagnosis Chorionic Villous Sampling (CVS) was done in one of the pregnant mother of an index case, but as SCK analysis could not be carried out on CVS so it was also excluded from this study.

DNA extraction of each family was carried out in separate batches by using Puregene DNA Purification system/blood kit by GENTRA system. The DNA extraction of CVS was done in duplicate by Phenol Chloroform Extraction Technique.[14] After DNA extraction the Polymerase Chain Reaction for DMD was carried out with STR in the introns 44, 45, 49 and 50 of DMD gene, as described by Clemens and Chamberlain et al.[15] The Perkin Elmer Thermocycler was programmed for 25 cycles for DMD each comprising 30 sec denaturation at 94 °C, annealing at 62 °C for 30 sec, extension at 65 °C for 2 min and a final extension at 65 °C for 3 min. The amplified products were run on 8% non-denaturing Polyacrylamide gels and electrophoresis carried out at 200 V for 3 hours. The gels were stained in silver nitrate.

The Serum samples for Creatine Kinase were analysed in each case on Semi-automated Chemistry Analyser Microlab 300™ by International Federation of Clinical Chemistry and laboratory[16] (IFCC) recommended method with Teco Diagnostics Kit (USA). This method is based on the principle that SCK catalyses the conversion of Creatine phosphate and ADP to Creatine and ATP. The glucose and ATP are converted to ADP and glucose-6-phosphate by Hexokinase (HK). Glucose-6-phosphate dehydrogenase (G-6-PDH) oxidises the D-glucose-6-phosphate and reduces the Nicotinamide Adenine Dinucleotide (NAD). The rate of NADH formation, measured at 340 ηm is directly proportional to CK activity in the serum. Normal reference range considered for females and males was 34–145 IU/L and 46–171 IU/L at 37 °C respectively. Linearity of the assay was 1,200 IU/L. Two level controls were run with each batch. Results which were outside assay linearity range were rerun after appropriate dilution according to the recommendations of the manufacturer of the kit and reanalysis/interpretation of results were done accordingly.

Data were analysed using SPSS-11. Inferential statistics included frequency of occurrence of: carrier mothers and siblings and non carrier siblings, affected (both index cases and symptomatic males) and non-affected males based on clinical features and PCR analysis. Appropriate statistics were applied to summarise the data. Descriptive statistics were applied to analyse Mean, Standard Deviation (SD) and ranges for SCK in individuals of DMD families. PCR analysis with the informative Intron 44 was taken as 'gold standard' to determine the diagnostic efficacy of SCK in terms of sensitivity, specificity, and positive and negative predictive values by using standard formulae.

$$\text{Sensitivity} = \frac{\text{True Positive (TP)}}{[\text{True Positive} + \text{False Negative (FN)}]} \times 100$$

$$\text{Specificity} = \frac{\text{True Negative (TN)}}{[\text{False Positive (FP)} + \text{True Negative}]} \times 100$$

Positive predictive value (PPV) = TP/(TP+FP) ×100
Negative predictive values (NPV) = TN / (TN+FN) ×100
Efficiency of the test = TP+TN/(TP+FP+FN+TN) ×100

RESULTS

A total of 32 family members from six informative families were evaluated with the above mentioned methods. The PCR analysis with intron 44 had classified 13 females in DMD families into 10 carrier females and 3 non carriers. As enzyme analysis was not done in one carrier detected on Chorionic villous biopsy so this was excluded from the study. PCR analysis of one of the family is shown in Figure-1.

There were 6 (50%) carrier mothers, 3 (25%) carrier sisters, and 3 (25%) non-carrier sisters; and 8 (42%) affected, and 11 (58%) non-affected males in the study group. Out of nine carrier females, results of SCK were within reference range in all maternal carrier (73±37 U/L). In the three carrier siblings the SCK was 1032±611 U/L, and in non-carrier siblings the SCK was 44±30 U/L. (Table-1).

The overall sensitivity and negative predictive value of SCK were 33.3%, and specificity and positive predictive were 100% with diagnostic

efficiency of 50% regarding carrier detection in DMD families (Table-2).

In affected cases of DMD the sensitivity and negative predictive value of S.CK were 100%, and specificity and positive predictive were 91% and 88.8% respectively and diagnostic efficiency of 94.1% (Table-3).
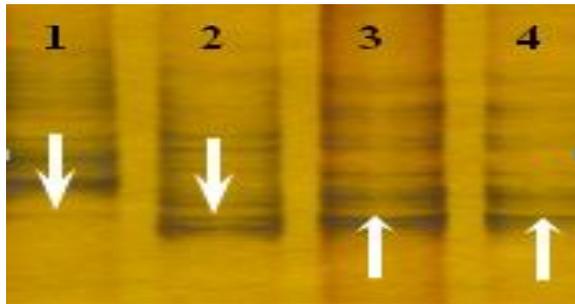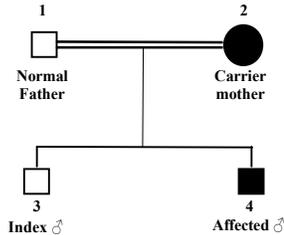

Figure-1: Poly Acrylamide Gel Electrophoresis (PAGE) of amplified STR products in intron 44 of DMD

Gene in one of the study family with two male children, the elder one was index case of DMD (lane 3) and the younger one (lane 4) (age one and a half year, still asymptomatic and presented with history of delayed walking) .The status of younger sibling regarding inheritance/non inheritance of DMD gene, was to be determined by PCR analysis. Analysis of PAGE showed, father's sample in Lane 1 and arrow indicated normal X-chromosome allele. Lane 2 showed mother's sample with two alleles on the X-chromosome. The lane 3 index child and arrow showed affected X-chromosome allele. Comparison of the results in Lane 3 (index child) and lane 2 (mother) suggested that out of the two maternal X-chromosome alleles, the upper was abnormal (pointed by arrow as this X-chromosome allele was inherited by index case) while the lower allele was normal. Analysis of sample in lane 4 showed that the younger sibling had inherited affected X-chromosome allele.

Table-1: Creatine Kinase analysis in females of DMD families (n=12)

Females of DMD families in the study	Creatine Kinase (IU/L)			
	No (%)	Mean±SD	Min	Max
Carrier mothers	6 (50%)	73±37	29	129
Carrier sisters	3 (25(%)	1032±611	345	1512
Non-carrier sisters	3 (25%)	44±30	20	78

Table-2: Enzyme analysis in females of six informative DMD families (n=12)

Results of tests (CK)	Carriers	Non-carriers	Total
TP	3	3	6
FP	0	6	6
Total	3	9	12

- Sensitivity= 3/9×100=33.3%,
- Specificity= 3/3×100=100%
- PPV= 3/3×100=100%
- NPV= 3/9×100=33.3%
- Diagnostic efficiency= 50%

Table-3: Enzyme analysis in affected and non-affected males of six informative DMD families (n=19)

Results of tests(CK)	Affected	Non affected	Total
TP	8	10	18
FP	1	0	1
Total	9	10	19

- Sensitivity=8/8×100=100%
- Specificity=10/11×100=91%
- PPV=8/9×100=88.8%
- NPV=10/10×100=100%
- Diagnostic efficiency=94.1%

DISCUSSION

Duchenne Muscular Dystrophy is characterised by progressive degeneration of skeletal muscles caused by the absence of dystrophin.[17] Currently no treatment is available for this disease that affects boys of all ethnic origins with an inheritance pattern of one case per 3,500 live male births.[18] In our setup the exact incidence of the disease is unknown. A study conducted at the Children's Hospital and the Institute of Child Health had documented Duchenne muscular dystrophy as the commonest diagnosis in 8/10 patients of muscular dystrophy based on histological diagnosis.[19]

Although the combination of different biochemical markers have been tried for diagnosis of carriers. Few studies had found positive correlation of combined markers, i.e., SCK and Carbonic anhydrase III in muscular dystrophy and neurological disorders.[20] However the differential diagnosis based on enzyme analysis is very wide[10] and one of the limitations is that their estimation is not reliable on CVS[5] because the methods are not standardised in every laboratory.

In our study the SCK had overall sensitivity and negative predictive value of 33.3% while in different studies the SCK levels were elevated in 50–60% of carriers[1], this difference can be because in our study 66% were maternal carriers with normal SCK analysis while all the three carrier female siblings had elevated SCK which clearly signifies the diagnostic dilemma of picking up of carriers with biochemical analysis of SCK. All the carrier siblings were less than 12 years of age which indicated that SCK level can be informative in the siblings in their early childhood but

with advancing age (as in maternal carriers) the reliability of SCK for carrier detection decreases. Our finding correlated with other studies[10,13] which had emphasised that enzyme analysis cannot be solely used as a reliable marker in carrier detection in DMD families and also emphasised the limitation of enzymes analysis and need for PCR based linkage analysis in DMD families. One of the limitations of this study was small sample size, so further studies in this regard are required.

In our study SCK was elevated in all index/ affected cases of DMD and correlated with the studies which supported SCK as highly specific and precise for DMD individuals.[10] As SCK has been employed in neonatal screening for DMD as a reliable indicator in many countries.[21] We can also use this simple marker to detect affected children in DMD families but further studies in this aspect are required to set the protocol of neonatal screening and to document the response of DMD families. In our study there was false positive elevation of SCK in a paternal sample possibly because he was muscular and tall.

CONCLUSION

The Serum Creatine Kinase is an excellent screening test for affected cases of DMD but for carrier identification we have to resort on PCR analysis so as to provide safer diagnostic tool for genetic counselling and prenatal diagnosis.

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