Screening of Dystrophin Gene Deletions in Malaysian Patients with Duchenne Muscular Dystrophy

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SUMMARY

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive genetic disorder characterized by rapidly progressive muscle weakness. The disease is caused by deletion, duplication or point mutation of the dystrophin gene, located on the X chromosome (Xp21). Deletion accounts for 60% of the mutations within the 79 exons of the dystrophin gene. Seven exons (43, 44, 45, 46, 49, 50, and 51) were found to be most commonly deleted among the Asian patients. To detect the frequency of deletion of these 7 exons in Malaysian DMD patients, we carried out a molecular genetic analysis in 20 Malaysian DMD patients. The mean age of initial presentation was 60 months (SD 32 months, range 5-120 months). Fourteen patients were found to have deletion of at least one of the seven exons. The remaining six patients did not show any deletion on the tested exons. Deletions of exons 49, 50 and 51 were the most frequent (71.43%) and appear to be the hot spots in our cohort of patients.

KEY WORDS:
Duchenne muscular dystrophy (DMD), Dystrophin gene, X-linked resessive

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a rapidly progressive form of muscular dystrophy and is considered as one of the most common lethal paediatric disorder worldwide. It is an X-linked inherited disorder with an incidence of 1 in 3500 live births. DMD is caused by mutations such as deletion, duplication or point mutation of the dystrophin gene that encodes for the protein dystrophin. DMD is usually diagnosed by measuring the serum creatine kinase (SCK) level, muscle biopsy, clinical examination and molecular genetic analysis for mutations of the dystrophin gene. The dystrophin gene is located on the short arm of the X chromosome (Xp21), encoding 79 exons (Biggar *et al.,* 2002). Dystrophin is one of the components of the plasma membrane cytoskeleton, which anchors and supports the sarcolemma during exercise. Due to its large size, mutations can occur anywhere along the length of the gene. The severity of the disorder depends on the type of mutation, its location in the gene and the impact on production of functional dystrophin. Deletion of single or multiple exons within the dystrophin gene in DMD are responsible in about 65-70% of the cases; the remaining are point mutations (30%) or duplications (6%) (Emery, 2002). Among the deletion occurring in dystrophin gene, seven exons (43, 44, 45, 46, 49, 50 and 51) were documented to be more frequent in Asian DMD patients (Roddie *et. al.,* 1992). In this study, we screened the deletion frequencies of exons 43, 44, 45, 46, 49, 50 and 51 of the dystrophin gene in a group of Malaysian patients with DMD.

MATERIALS AND METHODS

Patients

Twenty male patients with a clinical diagnosis of DMD were referred to Human Genome Centre, Universiti Sains Malaysia, from various hospitals in Malaysia for molecular genetic analysis of dystrophin gene mutation. Clinical diagnosis was based on physical examination, progressive muscle weakness, muscle strength, high level of serum CPK, calf hypertrophy, positive Gower's Sign and myopahic changes on electromyography (EMG) and in some cases muscle biopsy (Table II). The patients comprised 13 Malays, 4 Indians, 2 Chinese and one other race from Sarawak.

Deletion Analysis

Blood samples were collected from these 20 clinically diagnosed DMD patients. Genomic DNA was extracted from whole blood using GeneAll Blood Mini kit (GeneAll, USA). Polymerase chain reaction (PCR) analysis of different exons of the dystrophin gene was performed using seven selected exons, which were exons 43, 44, 45, 46, 49, 50 and 51. The primer sequences and the expected size of PCR product for all of the tested exons are shown in Table I.

Amplification was performed using 1X PCR Buffer, 1.5mM $MgCI_2$, 0.2mM dNTP, 1pmol of primer (F/R) and 0.75U of Taq Polymerase (Promega, USA), ~40-100ng of DNA template and ddH_2O. The amplification conditions were denaturation 94°C, annealing 58°C, extension 72°C and final extension 72°C for exons 43, 44, 45, 46, 50 and 51. For exon 49, the conditions were similar except for the annealing temperature which was 60°C. The PCR products were visualized via UV by SYBR Green post staining after 2% agarose gel in 1X LB buffer electrophoresis.

Deletions were identified by the presence of a band in the amplified control DNA and the absence of the corresponding band in the patient's DNA (Figure 1).

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RESULTS

The mean age of patients at the time of initial presentation was 60 months (SD 32 months, range 5-120 months). Among the 20 DMD patients, 9 (45%) had a family history of the disease. There was no previous family history of the disease in 11 (55%) patients. Deletion analysis of the selected exons (43, 44, 45, 46, 49, 50 and 51) showed a deletion frequency of 70%. The remaining six patients did not have any deletion on the tested exons (Table III). Exons 49, 50 and 51 were found to be the most frequently deleted exons (71.4%). Six out of the 14 patients with deletion of the DMD gene, showed a positive Gower sign and family history (Table II). Four patients showed a positive Gower sign, two patients had positive family history and two patients showed negative Gower sign and family history. Six of the patients with deletion of the DMD gene were not investigated for the SCK level, only one had a normal SCK level and one showed a positive result for muscle biopsy. The remaining six had high SCK level (ranging from ~746 to 13000u/L). In the six patients without an identified deletion of the DMD gene, one had shown a high SCK level (4084u/L) and a positive Gower sign and family history (Table II). Four patients have positive Gower sign no the family history. One of the non-deleted patients did not have a positive Gower sign and family history. However, his SCK level was high (8000u/L). The SCK levels for these non deleted patients ranged between ~972 to 15000u/L.

DISCUSSION

Duchenne muscular dystrophy is caused by mutations in the dystrophin gene located on the short arm of the X chromosome. It is inherited in an X linked recessive manner and usually affects only males (Nishino *et al.,* 2002). Females are rarely affected; however females with an X-autosome translocation that disrupts the dystrophin gene, may also show the same phenotype. The patients who were referred to our centre and included in the present analysis were all males. Racial differences in incidence rate of DMD have been suggested (Roddie & Bunday, 1992). We have observed a higher incidence of causes in Malaysian races (65%) followed by Indians (20%), Chinese (10%) and other races (5%). However, considering the fact that Malaysian population consists predominantly of Malay races, it appears logical to have a higher incidence among Malay races.

Among the early warning in boys with DMD include delayed walking, waddling gait, and never being able to run properly, walking unsteadily with a tendency to fall easily, toe walking, difficulty in climbing stairs, muscle weakness, enlarged calves and the inability to rise from the floor without using the arms (*Emery,*1993).

The precise age at onset is difficult to assess. In our study, the mean age of patients at the time of initial presentation was about 5 years with a range of 5 months to 10 years. This is an older age compared to the previous report of Thong *et al.*,, who reported a mean age of 3 years and 8 months with a range of 10 months to 7 years.

In our study, only one out of the 20 patients had shown a positive muscle biopsy result while the remaining patients never underwent a muscle biopsy (Table II). The probable reason for this is the refusal for the invasive procedure and the availability of non invasive tests such as molecular genetic analysis. If a mutation is not detected by direct molecular analysis, a linkage analysis might be required to assess the carrier risk. These advances have reduced the number of boys requiring a muscle biopsy for diagnosis and have greatly facilitated carrier detection for genetic counseling.

Among the 20 patients that we have screened, patients 1 and 2 were brothers, of which patient 1 is the elder brother (Table II). Both patients have deletion in all the seven exons tested. The first symptom of DMD appeared in patient 1 at the age of 12 months and for patient 2 at the age of 19 months. However, only the elder brother had a positive Gower sign. These results are in agreement with a previous report by Ayfer *et al.,* (2004), which concluded that there was no correlation between the walking age and the localization of deletion. Other reports by Bodrug *et al.,* (1990) and Iwanczak *et al.,* (2000), observed that children with Duchenne or Becker dystrophinopathy may walk at a relatively normal age but a delay in standing independently may be recognizable.

Bakker *et.al.,* (1987) reported that in one-third of the cases, the disease is the result of a *de novo mutation* in the DMD gene. They reported two DMD families with an apparent *germline mosaicism,* i.e. a somatic non-carrier mother who transmited a de novo mutation more than once (consequently several of her germ cells carry the mutation). Since our samples were received from various hospitals, we were not able to test the mothers for their carrier status.

The oldest patient referred to us was at the age of 16 years (patient 19). He had no deletion within any of the tested exons. However, he showed a positive Gower sign and a high SCK level. It is presumed that although he did not have a deletion in the seven tested exons, he might have a mutation in any of the remaining exons. This situation is similarly reflected in patient 6. Our youngest patient was a five month old boy (patient 13) who had deletion on exons 49, 50 and 51. He also has a strong family history of DMD.

Our study showed that 14 out of 20 DMD patients (70%) had deletions. For accurate comparisons of deletion frequencies, larger patient populations should be included and the patients must be confirmed to have DMD either by muscle biopsy or classical clinical presentations to rule out false disease pick-up of other similar muscle disorders. The 7 exons were selected for this study as they are in the distal mutation hot-spot of the DMD gene. Within this deletion-prone region, our results show that exons 49, 50 and 51 are most commonly deleted. Hence, in cases where there is consideration of urgency or cost of testing, these 3 exons could be initially screened to rule out the common mutation and proceed to other exon screening if the initial analysis proved negative. Future work for our study would include extending the studies to more exons, screening for other mutation for the non-deleted patients and multiplexing the reactions so that more than one exon can be analyzed simultaneously and enlarging the patient sample size for a more extensive molecular study on DMD patients in Malaysia.

Table I: 'Hotspot' exons, primer sequences and the expected size of PCR product for detection of homologous deletion in Duchenne Muscular Dystrophy

Exon	Sequence	Expected size of PCR product
43	F: 5'-CACCATTTGCTACCTTTGGG-3'	
	R: 3'-TTCAGCTCATTTGTCTGAATTG-5'	455bp
44	F: 5'-TTGTGTGTACATGCTAGGTGTG-3'	
	R: 3'-CCAGGAAACTCTCTCATCC-5'	541bp
45	F: 5'-GGGAAATTTTCACATGGAGC-3'	
	R: 3'-CCTTTAAGCAATCATGGGTGA-5'	571bp
46	F: 5'-GTGTCCCAGTTTGCATTAAC-3'	
	R: 3'-TCTTATGTGCACTGGTTCAG-5'	519bp
49	5'-GCCCTTATGTACCAGGCAGA-3'	
	3'-GAGTCCTTTAAAGCAATGACT-5'	449bp
50	5'-TATTTGTAGGGTGGTTGGCT-3'	
	3'-CCGTTGTCATGCAACACTTT-5'	490bp
51	5'-TCATGAATAAGAGTTTGGCTCA-3'	
	3'-TTAGGCTGAATAGTGAGAGTAATGTG-5'	522bp

Table II: Clinical characteristics in 20 Malaysian DMD patients

Patient	Ethnicity	SCK level	Onset Age (Month)	Present Age (Month)	Gower Sign	Family History	Mutation status of 7 tested exons
1	CHN	NA	12	96	YES	YES	Deletion
2	CHN	NA	19	48	NO	YES	Deletion
3	MLY	NORM	24	72	NO	NO	Deletion
4	MLY	746	60	144	YES	NO	Deletion
5	IND	NA	48	96	YES	YES	Deletion
6	IND	4084	60	96	YES	YES	No
7	MLY	NA	108	108	YES	YES	Deletion
8	MLY	5798	60	96	YES	YES	Deletion
9	MLY	3338	96	108	YES	YES	Deletion
10	MLY	972	48	84	YES	NO	No
11	MLY	8880	84	96	YES	NO	Deletion
12	MLY	NA	48	60	YES	YES	Deletion
13	MLY	NA	5	5	NO	YES	Deletion
14	MLY	7952	120	132	YES	NO	No
15	MLY	8000	60	84	NO	NO	No
16	MLY	MBP	NA	84	NO	NO	Deletion
17	IND	15260	84	84	YES	NO	No
18	OTH	11770	72	84	YES	NO	Deletion
19	MLY	HIGH	NA	192	YES	NO	No
20	IND	12170	72	108	YES	NO	Deletion

MLY: Malay, CHN: Chinese, IND: Indian, OTH: Other, NA: Not Available, NORM: Normal and MBP: Muscle Biopsy Positive

Table III: Deletion status of the 7 exons in 20 Malaysian DMD patients

Patient	EXON						
	43	44	45	46	49	50	51
1	del	del	del	del	del	del	del
2	del	del	del	del	del	del	del
3	non	del	non	non	non	non	del
4	non	non	non	del	del	non	del
5	non	del	non	non	del	del	non
6	non	non	non	non	non	non	non
7	non	del	del	non	non	non	del
8	non	non	non	del	del	del	del
9	non	non	del	del	del	del	del
10	non	non	non	non	non	non	non
11	non	non	non	del	non	non	non
12	non	non	non	non	del	del	del
13	non	non	non	non	del	del	del
14	non	non	non	non	non	non	non
15	non	non	non	non	non	non	non
16	non	non	non	del	del	del	non
17	non	non	non	non	non	non	non
18	non	non	non	non	non	del	non
19	non	non	non	non	non	non	non
20	non	non	del	del	del	del	del
TOTAL	2	5	5	8	10	10	10

DEL: deleted and NON: Non-deleted

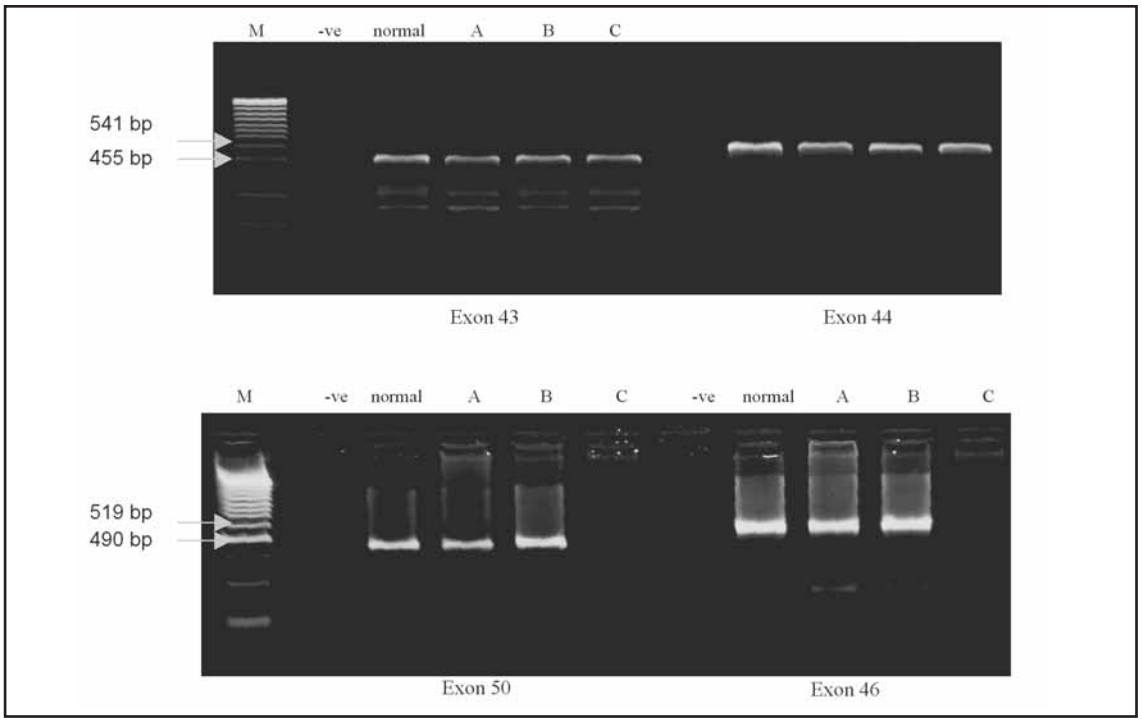

Fig. 1: PCR analysis: dystrophin gene deletion encompassing exons 46 and 50. (M: marker, -ve: negative PCR, normal: normal sample, (A, B, C): patient with DMD)

CONCLUSION

In this study, deletion of exons 49, 50 and 51 emerged as the most frequent deletions. Based on this preliminary finding, it is reasonable to presume that these exons might be the 'hot spots' of DMD within the Malaysian population. A larger study with more samples is currently in progress to confirm these findings. Identification of 'hot spots' would facilitate rapid initial analysis and reduce cost for genetic test as the more commonly deleted exons could be screened first among the patients in our population. This is especially important since the DMD gene is the largest known in the human genome and there are at least 79 exons and other promoter and regulatory regions which can be mutated in causing the disease.

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