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DUCHENNE AND BECKER MUSCULAR DYSTROPHY

A molecular and immunohistochemical approach

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ABSTRACT - Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by mutations in the dystrophin gene. We studied 106 patients with a diagnosis of probable DMD/BMD by analyzing 20 exons of the dystrophin gene in their blood and, in some of the cases, by immunohistochemical assays for dystrophin in muscle biopsies. In 71.7% of the patients, deletions were found in at least one of the exons; 68% of these deletions were in the *hot-spot* 3' region. Deletions were found in 81.5% of the DMD cases and in all the BMD cases. The cases without deletions, which included the only woman in the study with DMD, had dystrophin deficiency. The symptomatic female carriers had no deletions but had abnormal dystrophin distribution in the sarcolemma (discontinuous immunostains). The following diagnoses were made for the remaining cases without deletions with the aid of a muscle biopsy: spinal muscular atrophy, congenital myopathy; sarcoglycan deficiency and unclassified limb-girdle muscular dystrophy. Dystrophin analysis by immunohistochemistry continues to be the most specific method for diagnosis of DMD/BMD and should be used when no exon deletions are found in the dystrophin gene in the blood.

KEY WORDS: Duchenne muscular dystrophy, Becker muscular dystrophy, immunohistochemistry, PCR, deletions, exons.

Distrofia muscular de Duchenne e Becker: abordagem molecular e imuno-histoquímica

RESUMO - As distrofias musculares de Duchenne (DMD) e de Becker (DMB) são doenças causadas por mutação no gene da distrofina. Foram estudados 106 casos com a suspeita diagnóstica de DMD/BMD com a analise de 20 exons do gene da distrofina no sangue e biópsia muscular com imuno-histoquímica para distrofina em alguns casos. Em 71,7% dos casos foi encontrada deleção em pelo menos um dos exons, sendo que 68% das deleções localizam-se na região 3´ *hot spot.* Foram encontradas deleções em 81,5% dos DMD e em todos os BMD, sendo que os sem deleção tinham deficiência de distrofina, incluindo a mulher com DMD. As portadoras sintomáticas não tinham deleções mas anormalidades na distribuição da distrofina no sarcolema. Os outros casos sem deleção, com auxilio da biópsia muscular tiveram outros diagnósticos (atrofia muscular espinhal, miopatia congênita, deficiência de sarcoglicanos, distrofia de cinturas-membros sem classificação). A análise imuno-histoquímica para distrofina na biópsia muscular continua sendo o método mais específico para diagnóstico de DMD/DMB e deve ser utilizado quando não são encontradas deleções do gene da distrophina no sangue.

PALAVRAS-CHAVE: distrofia muscular de Duchenne, distrofia muscular de Becker, imuno-histoquímica, PCR, deleções, exons.

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are progressive neuromuscular diseases caused by mutations in the dystrophin gene at the Xp21 locus[1,2]. This gene spans a 2.4 Mb region containing 79 exons and produces a 14 Kb mRNA[3,4] which encodes a large cytoskeletal protein (427 kD)[5]. This protein, called dystrophin, is located on the inner face of the plasma membrane of all types of myofibers[2,6-8]. The mutations in the dystrophin gene that are responsible for DMD/BMD disorders can be large deletions, partial duplications or point mutations[3,9,10]. There is no correlation between mutations and severity of the disorder. The severe Duchenne form of muscular dystrophy is the result of mutations that alter the translational reading frame, leading to production of a truncated and presumably unstable dystrophin molecule, and this deficient protein is found in these patients. The milder BMD, on the oth-

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er hand, is caused by internal mutations that do not disrupt the reading frame, so partially functional protein can still be produced[11,12]. In the past, deletions and duplications of the dystrophin gene were detected by Southern-blot analysis using cDNA probes[9,10,13]. Nowadays, deletions are detected in many DMD/BMD patients by a series of PCR assays[14].

DMD and BMD are characterized by an X-linked pattern of inheritance, and thus affect mainly males. However, they have also been reported in females, in cases of skewed X-inactivation or other X-chromosome abnormalities. In these cases there are differences in the intensity of the symptoms[15].

We report the frequency of dystrophin gene deletions in a group of patients with clinical evidences of DMD/BMD.

METHOD

We studied 106 unrelated patients referred for DNA analysis of the dystrophin gene between 1999 and 2005. Most had a clinical diagnosis of DMD or BMD based on clinical findings, electromyography (EMG) and elevated serum creatine kinase activity. The consent to realize the muscle biopsy and DNA tests was obtained in the out-patient clinic or during hospital admission for diagnostic investigation.

DNA analysis – The DNA was isolated from peripheral blood leukocytes using the standard phenol/chloroform method[16]. PCR analysis was performed using primers previously described. An isolated reaction was carried out for each of the 20 exons selected[14,17,18]. The multiplex reactions were not performed because the isolated one is more credible and compatible with the conditions of our laboratory. The exons studied were numbers 3, 4, 6, 8, 12, 13, 17, 19, 42, 43, 44, 45, 47, 48, 50, 51, 52 ,53, 60 and Pm. The PCR products were analyzed on a 7% polyacrylamide gel and the bands visualized by silver staining[19].

Muscle biopsy – The muscle biopsy was done in fifty one patients. Some had the DNA analysis before (normal) or during the work-up investigation (simultaneously muscle biopsy and blood draw for DNA analysis). These fifty one samples were freshly frozen and cut on cryostat into 8-micron sections and stained with hematoxylin-eosin, modified Gomori trichrome, oil-red O, PAS, cresyl violet and

Table 1. Frequencies of deletions in patients in 20 exons analyzed in the 5' hot spot and 3' hot spot regions.

Exons	Frequencies - %	
	5' hot spot	3' hot spot
03	8.33	
04	2.38	
06	5.95	
08	3.57	
12	4.76	
13	5.95	
17	7.14	
19	13.10	
42		2.30
43		11.9
44		5.95
45		19.05
47		3.57
48		4.76
50		10.71
51		7.14
52		7.14
53		8.33
60		1.19
PM		1.19

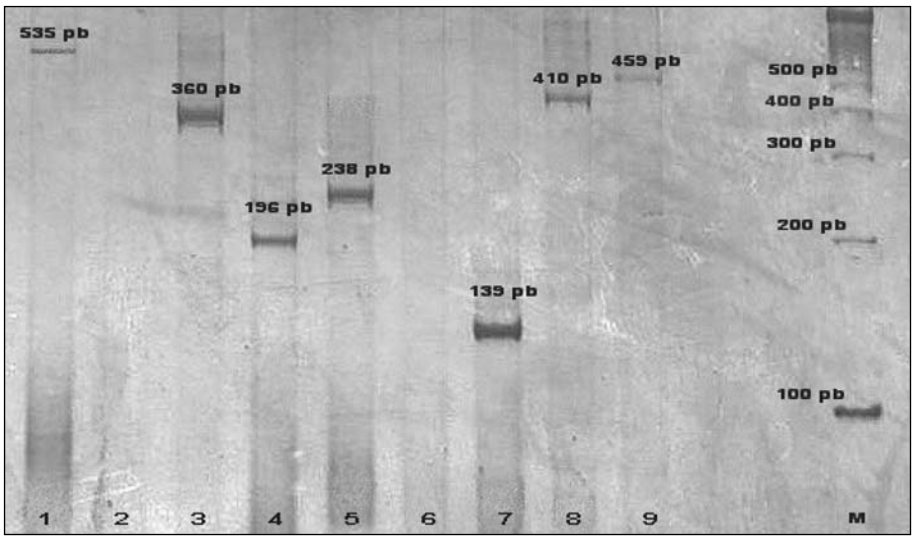

Figure. The PCR products of exons 3, 4, 8, 13, 19, 43, 45, 60 and Pm on a 7% polyacrylamide gel. Bands visualized by silver staining. This patient had deletions in exons 43 and 45. Line 1: promoter/535 bp; Line 2: deletion in exon 45/ 307 bp; Line 3: exon 8/ 360 bp; Line 4: exon 4/ 196 bp; Line 5: exon 13/ 238 bp; Line 6: deletion in exon 43/ 357 bp; Line 7: exon 60/ 139 bp; Line 8: exon 03/ 410 bp; Line 9: exon 19/ 450 bp and M: ladder 100 bp.

Table 2. Findings of molecular and dystrophin analysis in 106 patients with suspected DMD/BMD.

	PCR		Dystrophin analysis		
	Tested	Deletion	Tested	Absence	Absence in at least one of the three domains or discontinuous dystrophin immunostains in the sarcolemma
Male DMD	80	66	34	34	
Female DMD	1	0	1	1	
Male BMD	10	10	1		1
Female carriers	3	0	3		3
Unclassified limb-girdle muscular dystrophy	7	0	7	0	
Spinal muscular atrophy	2	0	2	0	
Sarcoglycanopathy	2	0	2	0	
Congenital myopathy	1	0	1	0	
Total	106	76	51	35	4

DMD, Duchenne muscular distrophy; BMD, Becker muscular distrophy; PCR, polymerase chain reaction.

Sirius red. They were then processed with ATPase pH 9.4, 4.3 and 4.6, myophosphorylase, non-specific esterase, NADH-tetrazolium reductase, succinic dehydrogenase, cytochrome c-oxidase and acid and alkaline phosphatase[20]. Afterwards, the biopsies were cut into 4-micron sections and submitted to immunohistochemistry for dystrophin proteins (rod and carboxyl- and amino–terminal domains), sarcoglycans (alpha, beta, gamma and delta) and dysferlin[21-24].

RESULTS

We detected at least one deletion in the 20 dystrophin-gene exons analyzed in 76 cases (71.7%) (Fig 1).

A total of 121 deletions were found in these 76 cases. Eighty-two deletions (68%) were detected in exons 42 to 60 (3´ hot-spot); 39 deletions (32%) were detected in exons 3 to 19 (5´ hot spot). Fifteen patients shared deletions in both hot-spot regions. The most frequent deletions found in these patients were in exons 45 (19.05%), 19 (13.10%) and 43 (11.9%) (Table 1).

We detected deletions in 10/10 male BMD patients and 66/80 male DMD patients; of the total of 106 cases, 30 (including a female DMD patient and 3 symptomatic female carriers) had normal PCR results. These cases with normal PCR were submitted to muscle biopsy, as were other cases, for which exon deletions had been detected.

The diagnosis of DMD or BMD was confirmed by dystrophin analysis in 39/51 muscular biopsies, 21/39 (53%) of which had deletions in the dystrophin gene. One symptomatic carrier showed an absence of the carboxyl region of dystrophin, and 35 DMD patients, including one female, showed no reaction for the carboxyl- and amino-terminal and rod domains. Only one BMD patient and two symptomatic female carriers had large gaps or punctuated discontinuous immunostains for dystrophin in the sarcolemma of muscle fibers. Twelve of the fifty-one muscle biopsies analyzed showed the presence of other muscle diseases. Two had severe denervation in the muscle biopsy compatible with spinal muscular atrophy (SMA); one had alterations in muscular fibers compatible with congenital myopathy; two had sarcoglycan deficiency; and seven had alterations compatible with muscular dystrophy but had normal immunohistochemistry (unclassified limb-girdle muscular dystrophy) (Table 2).

Of the 106 patients, 18 (including two symptomatic female carriers), or 17% of all the cases, had a family history suggestive of muscular dystrophy.

DISCUSSION

We studied 20 exons of the dystrophin gene and detected deletions in 71.7% of the patients. A large number of these deletions were detected in the 3´ extremity. Deletions in the patients studied were most frequent in exons 45 and 43. All these findings are similar to those described by other authors[25-27].

The deletion frequency of 71.7% is different to that found previously in another study[27], in which DNA was extracted from impaired muscular tissue. In the present study, however, we used DNA extracted from leukocytes. We found similar frequencies to those described in the current literature[14,17,18,25]. It is

possible that by using leukocytes we were not able to find deletions caused by somatic mosaicism or germ-line cells[27].

Thirty samples for which DNA deletions were not found in blood were submitted to muscle biopsy and immunohistochemistry for dystrophin, sarcoglycans and dysferlin. This showed alterations in muscle fibers, such as severe denervation, congenital myopathy, unclassified limb-girdle muscular dystrophy and dystrophin or sarcoglycan deficiency. Thus, immunohistochemistry of muscle biopsy is still the gold-standard for diagnosis of DMD/BMD but is a more invasive procedure and should only be performed in those cases where PCR blood screening does not detect deletions. To spare more patients from muscle biopsy, other techniques to investigate mutations and other methodologies, such as quantitative real-time PCR[28], should be used, particularly when the patient symptoms, family history and clinical findings suggest DMD or BMD.

Of the 106 patients, 17% had a family history of DMD/BMD. Given this level of family history, the detection of any mutations in the patient's family provides accurate information for genetic counseling and allows prenatal diagnosis to be proposed for female carriers. For patients who do not have a family history of DMD/BMD mutations, linkage analysis using polymorphic markers is still the only option proposed for females at risk[26].

The three symptomatic female DMD carriers had their diagnosis confirmed by dystrophin analysis. Two carrier patients had DMD cases in their family. None of the four women in the sample with abnormal dystrophin analysis showed any DNA deletions. Our failure to find deletions in female patients is probably a consequence of the methodology used, as the amplification endpoint was analyzed and there is some correlation with the amount of initial DNA used. In most cases, symptomatic carrier women present deletions of exons in only one of the X chromosomes; therefore, if the same exon is normal in the other chromosome, amplification will occur. The female with DMD who presented normal karyotype which was not characteristic of Turner's syndrome could have a deletion or mutations in other exons not analyzed in this study.

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