Primary adhalinopathy: a common cause of autosomal recessive muscular dystrophy of variable severity

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Marked deficiency of muscle adhalin, a 50 kDa sarcolemmal dystrophin-associated glycoprotein[1-3], has been reported in severe childhood autosomal recessive muscular dystrophy (SCARMD)[4]. This is a Duchenne-like disease affecting both males and females first described in Tunisian families[5,6]. Adhalin deficiency has been found in SCARMD patients from North Africa[4,7,8] Europe[9], Brazil[10], Japan[11,12] and North America (SLR & KPC, unpublished data). The disease was initially linked to an unidentified gene on chromosome 13 in families from North Africa[7,13], and to the adhalin gene itself on chromosome 17q in one French family in which missense mutations were identified[14]. Thus there are two kinds of myopathies with adhalin deficiency: one with a primary defect of adhalin (primary adhalinopathies), and one in which absence of adhalin is secondary to a separate gene defect on chromosome 13. We have examined the importance of primary adhalinopathies among myopathies with adhalin deficiency, and describe several additional mutations (null and missense) in the adhalin gene in 10 new families from Europe and North Africa. Disease severity varies in age of onset and rate of progression, and patients with null mutations are the most severely affected.

We examined a total of 12 new independent cases of autosomal recessive muscular dystrophy from various origins (France, Italy, Germany, Algeria, Morocco). Five were familial cases; seven were sporadic. In all patients proximal muscles were predominantly affected and serum creatine-kinase levels were elevated, but the clinical pattern was of variable severity, ranging from severe childhood progressive muscular dystrophy to very mild forms with late-onset and minimal muscle impairment (Table 1). Intellectual development was normal in all cases. There was no heart dysfunction except in one family (#13) where two male siblings exhibited severe heart failure. In all patients but one (#2) muscle biopsy was performed, showing a typical necrosis-regeneration pattern of the muscle fibres, with variable degrees of the dystrophic process and connective tissue proliferation. In all these patients dystrophin was normal, whereas adhalin was deficient as Judged by immunostaining of cryosections or western blot analysis. The deficiency varied from total absence of signal to some residual adhalin staining of normal size by western blot analysis (Table 1, and Fig. 1).

Linkage analysis was performed in one family from Algeria (#1) and one from Morocco (#2). Negative lodscores were obtained with the markers for the SCARMD locus on chromosome 13q and positive scores for the markers of the 17q21 region including the intragenic adhalin microsatellite[15] (data not shown). Mutation analyses were performed in patients from the two adhalin gene-linked families from North Africa, and in 10 European cases where linkage analysis was not possible: five from France (one familial and four sporadic), three sporadic cases from Italy and two familial cases from Germany (Table 1). The adhalin gene was explored by PCR amplification of genomic DNA sequences corresponding to exons 1 to 8 and their flanking intronic sequences (representing altogether more than 95% of the coding sequence), followed by denaturing gradient gelelectrophoresis (DGGE) analysis and direct sequencing. In all cases the parental origin of the mutation (s) was assessed and the segregation among the siblings established.

We found a variety of mutations in 18 out of the 24 chromosomes from the 12 new families explored, excluding the previously reported family #7 (refs 14,16) (Tables 1 & 2). In two patients (#12 and #13), no mutation was found. In these cases mutations may be located in the unexplored coding and non-coding sequences of the adhalin gene. Alternatively they may represent secondary adhalinopathies.

Among the ten new families with a mutated adhalin gene, four patients were homozygous: an inbred family from Morocco (#2), family #l from Algeria with suspected consanguinity, and two European families without evidence of consanguinity, one from Germany (family #4), and one from Italy (family #6) (Table 2). In seven families the patients were compound heterozygotes. In one families (#11) only one abnormal allele was found.

Altogether, including the first reported family[14] (#7), adhalin mutations were found on 21 chromosomes — two carried a nonsense mutation, two a duplication of 8 bp, three splice mutations affecting a critical G of an acceptor splice site, and 14 a missense mutation (Table 2). All substituted amino acid

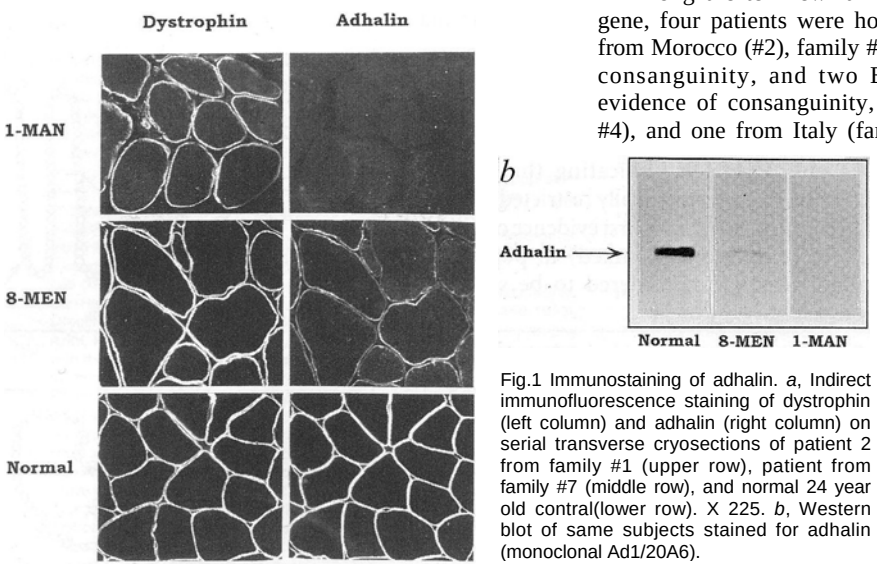

Fig.1 Immunostaining of adhalin. *a*, Indirect immunofluorescence staining of dystrophin (left column) and adhalin (right column) on serial transverse cryosections of patient 2 from family #1 (upper row), patient from family #7 (middle row), and normal 24 year old contral(lower row). X 225. *b*, Western blot of same subjects stained for adhalin (monoclonal Ad1/20A6).

Table 1 Clinical presentation and adhalin status in 13 families with autosomal recessive muscular dystrophy

Family #	Origin	Number and sex of blot patients	Clinical onset	Age at loss of walk	Age in 1994 (clinical grade[a])	Creatine kinase (age at test)	Adhalin[b]	
							Immuno-flouresence	Western blot
1-MA	Algerian	1:M	6y	13y	20 y (10)	×4 (20y)	0	0
		2:F	6 y	14 y	19 y (10)	×8 (19 y)	0	0
2-HA	Moroccan	1:F	9 y	22 y	30 y (10)	Elevated	Not done	Not done
		2:M	7y	10y	28 y (10)	Elevated	Not done	
3-CH	French	1:M	9 y	22y	13 y (7)	×100 (12 y)	0	Not done
4-BE	German	1:M	4 y	No loss	12 y (3)	×70 (12 y)	0-1	Not done
		2: M	2y	No loss	10 y (2)	×90 (9 y)	0-1	Not done
5-GA	French	1:M	8 y	21y	24 y (6)	×32 (15y)	0/+	+
6-PE	Italian	1: F	7 y	No loss	17 y (5)	×93 (9 y)	0/+	0/+
7-HE[c]	French	1:F	12 y	No loss	15 y (3	×24 (14y)	0	+
		2:F	12 y	No loss	15 y (3)	×24 (14y)	0	+
		3:M	9 y	No loss	13 y (5)	×55 (12y)	0	+
		4:F	None	No loss	8 y (0)	×65 (7y)	0	+
		1:F	None	No loss	7 y (0)	×83 (6y)	0	+
8-ME	French	1:M	8 y	No loss	36 y (4)	×18 (27y)	+/++	+
9-BER	Italian	1:F	12 y	No loss	25 y (3)	×27 (25y)	+	+
10-BA	French	1:F	12 y	No loss	25 y (3)	×30 (20y)	++	+
11-JO	French	1:F	12 y	No loss	33 y (2)	×15 (30y)	++	++
		2:F	15 y	No loss	31 y (2)	×17 (28y)		
12-PA	*Italian*	*1:F*	*6y*	*No loss*	*14 y (5)*	*×49 (7 y)*	*0/+*	*++*
13-LU	*German*	*1:M[d]*	*6 y*	*10 y*	*At 11 y*	*×76 (10 y)*	*Not done*	*Not done*
		2:M[d]	*6 y*	*No loss*	*10 y (3)*	*×49 (10 y)*	*0/+*	*+*

Italics, cases in which no mutation was found in the adhalin gene.Families arranged in order of decreasing severity. Italics, cases in which no mutation was found in the adhalin gene. [a]Scored according to Walton's scale from 0 (no physical symptoms) to 10 (confined to bed)[20]. [b]Intensity of immunostaining was visually estimated (normal signal scored +++++). [c]This family has already been reported[14,16]. [d]Case with prominent cardiomyopathy.

residues are conserved in rabbit and hamster adhalin except Val 175, which is Ile in rabbit[17] and hamster (SLR and KPC, unpublished data). Each of the missense mutations segregated as a mendelian recessive trait. None was found in 160 control chromosomes, ruling out the possibility of these being common polymorphisms. Two polymorphic sequence variations were also found: C<u>G</u>→C<u>A</u>, at +27 in intron 1, and isosemantic G<u>T</u>C→GT<u>T</u>=Val(311)Val in exon 7; (manuscript in preparation).

The positions of the disease-related mutations in the protein are depicted in Fig. 2. Four out of the eight different missense mutations lie in exon 3, which may be a mutation hot-spot. Four missense mutations were C→T transversions affecting a methylable C. The recurrence of a given mutation on two or more unrelated chromosomes was observed for three different missense mutations (Table 2). One of them, Arg77Cys, was seen in four apparently unrelated chromosomes from two French and one German family.

Our results demonstrate that primary adhalinopathies are not exceptional and are characterized by a broad spectrum of adhalin mutations, excluding a possible founder effect. They were found in patients from France, Germany, Italy, Algeria and Morocco, indicating that primary adhalinopathies are not geographically restricted. Our two North African families provide the first evidence of primary adhalinopathy (chromosome 17-linked) in populations where adhalinopathy was considered to be secondary because of linkage to chromosome 13 (refs 7,8,13). It will be important to determine the respective frequency of the two categories by genetic analysis, as there is no distinctive clinical, pathological or biological feature.

The clinical severity of primary adhalinopathies varies strikingly. The most severe clinical course was observed in patients in which adhalin was completely absent (Table 1 & Fig. 1) and who are homozygous for null mutations (Table 2), whereas missense mutations were observed in milder forms of variable severity. It is premature to define correlations between the nature or site of the mutation and phenotype, mostly because patients are often compound heterozygotes. The observed missense mutations caused a pronounced decrease of the amount of adhalin. Their possible impact on the level of transcript is currently being investigated. Missense adhalin mutations may also

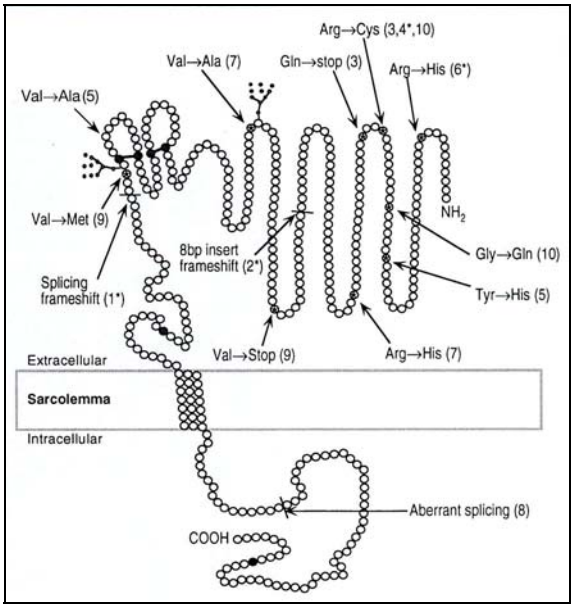

Fig. 2 Positions of mutations in the adhalin protein Each amino acid is represented by a circle (the first 23 amino acids, which comprise the signal sequence, have been removed). Conserved extracellular cysteines (grey circle), a potential Ca/CaM-dependent protein kinase phosphorylation site (blackcircle), and potential N-linked glycosylation sites (branched chains) are indicated. Stars indicate missense or nonsense mutations. Black bars indicate sites interrupted by splice mutations or insertion. Numbers refer to the patients listed in tables. Asteriks indicate homozygous mutations.

Table 2 Mutations in the adhalin gene

	Family #	Mutation and position in each allele	Consequences (codon number)
Severe phenotype	1-MA	AG→AA (-1 exon 7) AG→AA (-1 exon 7)	Aberrant splicing[a] Aberrant splicing[a]
	2-HA	Insertion of 8bp (exon 5) Insertion of 8bp (exon 5)	Frameshift Frameshift
	3-CH	CAG→TAG (exon 3) CGC→TGC (exon 3)	Nonsense (80) Arg77Cys[d]
Intermediate phenotype	4-BE	CGC→TGC (exon 3) CGC→TGC (exon 3)	Arg77Cys[d] Arg77Cys[d]
	5-GA	TAC→CAC (exon 3) GTT→GCT (exon 6)	Tyr62His Val242Ala
	6-PE	CGT→CAT (exon 2) CGT→CAT (exon 2)	Arg34His Arg34His
Moderate phenotype	7-HE[c]	CGT→CAT (exon 3) TCA→CCA (exon 5)	Arg98His[d] Val175Ala
	8-ME	CGT→CAT (exon 3) AG→AT(-1 exon 8)	Arg98His[d] Aberrant splicing[b]
	9-BER	TCA→TGA (exon 5) GTG→ATG (exon 6)	Nonsense (151) Val247Met[d]
Mild phenotype	10-BA	CGC→TGC (exon 3) GGA→GAA (exon 3)	Arg77Cys[d] Gly68Gln
	11-JO	GTG→ATG (exon 6) (2nd mutation not found)	Val247Met[d]

[a]Expected skipping of exon 7 (out of frame). [b]Expected skipping of exon 8 (in frame). [c]This family has already been reported[4,16]. [d]Mutations observed in two or more unrelated families.

affect protein stability, either intrisically or by perturbing essential sites of interaction with other protein(s). We conclude that primary adhalinopathies are a significant cause of autosomal recessive myopathies and are not geographically widespread. They may be caused by numerous different mutations, and may elicit muscle dystrophy of variable severity.

Note added in proof: Since this paper was submitted we have found mutations on 25 additional patients' chromosomes. Noteworthy is the fact that of a total of 46 mutated chromosomes, 11 carry the Arg77Cys missense mutation in seven unrelated families (France, Germany, Basque country, USA) including four homozygous patients.

Methods

Morphological studies and immunostaining of cryosections. Biopsies were obtained from all patients listed in Table 1 except in family #2. Cryosections were studied by histochemical and immunochemical methods as described[4,9,16]. Monoclonal antibodies against adhalin ($IVD3_1$) (ref. 4) and dystrophin (NLC DYS2 and NLC DYS3, Novocastra) were used.

Immunoblot analysis. Crude homogenates from muscle biopsy samples were prepared and processed essentially as described[18], except for the electrophoresis which was carried out in a homogeneous 10% SDS-polyacrylamide gel. Nitrocellulose blots were probed for dystrophin (using monoclonal antibodies DYS1 and DYS2, Novocastra) and adhalin (using affinity-purified polyclonal antibodies against adhalin fusion protein G and H[17]), and monoclonal antibody Adl/20A6 (raised against fusion protein G[17]) kindly donated by L.V.B. Anderson. Antibodies were detected by enhanced chemoluminescence (ECL, Amersham).

Sequence analysis. Genomic DNA extracted from blood or lymphoblastoid cell lines was analysed by PCR using oligonucleotide primers flanking the intron-exon junctions of the adhalin gene. Exon 1: Forward-GTG TCT ATC CCA GAT TTG G; Reverse-ACC ACC CCT AAA TCC TGC CC. Exons 2 and 3: Forward- ACC ATT AAT AGG GGG CCT AA; Reverse-CTC AAT GAC CTG GA. Exons 4 and 5: Forward-GGC CTC CTA GGA GCA GCC CTA TG; Reverse-GAC CCT TGT TCT ATG CCC GCC. Exon 6: Forward-CCG CTT CCT CTC AGC CTT GG; Reverse-ATC CAC CAA GAA GTC ACG GT. Exon 7: Forward-TGT GCG CTAACCAGT GCA; Reverse-AGT GCC ACT GTG AGT CCC. Exon 8: Forward-CAG GAG GGC ATT GTG GAT A; Reverse-GCA TCA CCC TGG AGT TAT. Exon 9: Forward-GGA ACA CAG AGG AGC TGC GG; Reverse-TGT GGA CAG AGA AGG GAG GAT G. Sequences of other primers and PCR conditions are available from the authors. After purification, the amplified product was sequenced on both strands using the same primers used for PCR amplification by the DyeDeoxy™ chain terminator method following the manufacturer's protocols (Applied Biosystem). When the sequence change affected or created a restriction site, the PCR product was digested with the appropriate restriction endonuclease and analysed by agarose gel electrophoresis. Some mutations were also rechecked by ASO hybridization. DGGE analysis was performed using Lerman's algorithm and psoralen clamps as described[19].

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