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ORIGINAL INVESTIGATION

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Nemaline myopathy in the Ashkenazi Jewish population is caused by a deletion in the nebulin gene

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Abstract Nemaline myopathy (NM) is a neuromuscular disorder that is clinically diverse and can be attributed to mutations in any of several genes. The Ashkenazi Jewish population, which represents a relatively genetically homogeneous group, has an increased frequency of several genetic disorders and has been the beneficiary of genetic screening programs that have reduced the incidence of these diseases. The identification of individuals with NM in this population has prompted a study of its cause. Our study has revealed that five NM patients from five families bear an identical 2,502-bp deletion that lies in the nebulin gene and that includes exon 55 and parts of introns 54 and 55. The absence of this exon results in the generation of a transcript that encodes 35 fewer amino acids. An analysis of the gene frequency of this mutation in a random sample of 4,090 Ashkenazi Jewish individuals has revealed a carrier frequency of one in 108.

Introduction

Nemaline myopathy (NM) is a slowly progressive or non-progressive neuromuscular disorder characterized by muscle weakness and the presence of rod-shaped structures (nemaline bodies or rods) in affected muscle fibers (Conen et al. 1963; Shy et al. 1963; Wallgren-Pettersson and Laing 1996; North et al. 1997). The estimated incidence is two per 100,000 live births (Wallgren-Pettersson 1990); however, NM may be more common in some populations. A recent study has suggested an incidence of one in 500 in the Amish community (Johnston et al. 2000). NM can be caused by mutations of (1) the α-tropomyosin-3 gene (TPM3; Laing et al. 1995) mapping to chromosome 1q22-23 (Wilton et al. 1995), (2) the nebulin gene (NEB; Pelin et al. 1999) mapping to chromosome 2q21.1-2q22 (Pelin et al. 1997), (3) the α-actin gene (ACTA1; Nowak et al. 1999) mapping to 1q42 (Ueyama et al. 1995), (4) the troponin T gene (TNNT1; Johnston et al. 2000) located at 19q13.4 (Samson et al. 1992), and (5) the β tropomyosin gene (TPM2; Donner et al. 2002) located at 9p13.2–9p13.1 (Tiso et al. 1997). Transmittance of NM can be autosomal dominant or autosomal recessive. Mutations in TPM3 and ACTA1 occur in families in which NM is inherited in either an autosomal dominant or autosomal recessive manner (Laing et al. 1995; Nowak et al. 1999; Tan et al. 1999; Ilkovski et al. 2001; Ryan et al. 2001). Mutations in TPM2 cause NM to be inherited in an autosomal dominant manner (Donner et al. 2002), and mutations in NEB and TNNT1 cause NM to be inherited in an autosomal recessive manner (Pelin et al. 1999; Johnston et al. 2000).

Nemaline myopathy shows wide clinical variability. Patients are classified into different subtypes according to the age of onset and the severity of the disease (Wallgren-Pettersson and Laing 2000; Wallgren-Pettersson et al. 1999; Ryan et al. 2001; Sanoudou and Beggs 2001). The typical form and most common form of NM is characterized by the infantile onset of a slowly progressive or non-progressive weakness of facial, bulbar, and respiratory muscles and neck flexors. Weakness initially is primarily proximal with later distal involvement. The typical form of NM is most often the result of mutations in the NEB gene (Pelin et al. 1999).

A number of autosomal recessive conditions are known to occur among individuals of Ashkenazi Jewish descent. These include, for example, Tay Sachs disease, cystic fibrosis, Canavan disease, Bloom syndrome, and familial dysautonomia. Carrier screening programs have reduced the incidence of these diseases. This success has led to increased interest in screening for other genetic diseases

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present in this population. The presence of individuals with NM in this population has prompted a study of the genetic cause of this disease.

We report that a 2,502-bp deletion in the gene encoding nebulin is responsible for NM in five families of Ashkenazi Jewish descent. We further demonstrate that this mutation occurs in this population at a frequency of approximately one in 108.

Materials and methods

Subjects and population studied

Blood samples were collected with the approval of the Institutional Review Board of Fordham University. Five families of Ashkenazi Jewish descent were identified in which one or more children were diagnosed with NM. In one of the families, the parents were first cousins. In the remaining families, the parents were not known to be related. One parent in each of two families could identify a common ancestor, and the remaining families appeared to be unrelated.

Blood samples from 4,090 anonymous individuals of Ashkenazi Jewish descent, mostly from the New York metropolitan area and Israel, were derived from the Dor Yeshorim screening program (Ekstein and Katzenstein 2001).

DNA purification

DNA was purified from the blood of probands, family members, and anonymous donors participating in the Dor Yeshorim genetic testing program by using the QIAamp DNA Blood Kit (Qiagen), according to the manufacturer's directions.

Haplotype analysis

For a determination of the haplotypes of the probands and their families, polymerase chain reaction (PCR) amplification was performed on purified DNA with primers specific for the D2S2275 and D2S2299 polymorphic microsatellite markers as previously described (Hudson et al. 1995). The products generated were size-fractionated on a 6% denaturing polyacrylamide gel.

Reverse transcription/PCR analysis of whole-blood-derived RNA

RNA was isolated from whole blood of probands and their family members by using the PAXgene Blood RNA Kit (Qiagen). This RNA was subjected to reverse transcription/PCR (RT-PCR) with primers that were located along the cDNA sequence of NEB and that were designed to generate products 200–300 bp in length. RT-PCR was performed in 10-µl reactions by using the QuantiTect RT-PCR Kit (Qiagen) in the presence of α–^{33} P-dATP under the following cycling conditions: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 30 s. The resulting products were characterized on 6% denaturing polyacrylamide gels (for size) and on 5% non-denaturing polyacrylamide gels for single-strand conformation polymorphism (SSCP) analysis as previously described (Anderson et al. 2001).

Amplification of the exon 54–57 product was carried out as described above, except no radionuclide was used. Primers used were 5′-AGCATTCCTGATGCCATGGA-3′ (exon 54 primer) and 5′-CTTGCGAAAGCCTTCCTTG-3′ (exon 57 primer). RT-PCR products were fractionated on a 2% agarose gel.

DNA sequencing

DNA sequences were determined by the dideoxy chain termination method by using the AmpliCycle Sequencing Kit (Applied Biosystems).

PCR detection of the R2478_D2512del mutation

For detection of the R2478_D2512del mutation, purified DNA was subjected to PCR analysis (94°C for 5 min, then 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s) with the following primers: 5′-AGGGTAGTGCAGAACTGGGA-3′ (intron 54 primer), 5′-AGAAGCTTGGGACAAAGAC-3′ (exon 55 primer) and 5′-GCCTATTGATCTTGGACTTG-3′ (intron 55 primer). Amplification of the allele containing the NM-causing deletion with these primers yielded a 360-bp DNA fragment (product of the intron 54/intron 55 primers), whereas amplification of the wild-type allele yielded a 672-bp fragment (product of the exon 55/intron 55 primers). A fragment of 2,862 bp (product of the intron 54/intron 55 primers) was also generated from the normal allele but was not efficiently amplified and was therefore not seen because of the short extension time in the PCR protocol. The sizes of the amplified DNA were determined by fractionation on a 2% agarose gel.

Results

As NM was inherited in an autosomal recessive manner in the five Ashkenazi Jewish probands in this study, and, as the typical form of NM (diagnosis of these probands) is often the result of mutations in nebulin, we examined the haplotypes of the probands and their parents at the D2S2275 and D2S2299 polymorphic microsatellite markers located adjacent to the nebulin gene. This analysis clearly revealed that the parents of the probands all shared common markers and that all of the five probands were homozygous for the same haplotype (Table 1). This finding prompted a thorough analysis of the nebulin transcript by using overlapping sets of primers located along the cDNA, in RT-PCRs performed on RNA isolated

Table 1 Haplotype analysis was performed on DNA from five probands and their parents. Allele numbers were assigned by designating the largest product obtained on a polyacrylamide gel as "1" for each of the two markers (*F* father, *M* mother, *A* affected)

Marker	Family														
	1			2			3			4			5		
	F	M	A	F	M	A	F	M	A	F	M	A	F	M	A
D2S2299	7,10	2,10	10,10	4,10	10,10	10,10	7,10	2,10	10,10	2,10	2,10	10,10	1,10	3,10	10,10
D2S2275	1,1	1,10	1,1	1,7	1,9	1,1	1,1	1,9	1,1	1,9	1,7	1,1	1,7	1,9	1,1

from whole blood derived from one of the probands and the proband's parents. The 200–bp to 300-bp products generated were subjected to size and SSCP analyses as previously described (Anderson et al. 2001). Size fractionation of the amplified products revealed that primers that spanned exons 54–57 of nebulin and that would normally generate a 300-bp product generated a 195-bp fragment in the proband and both of the 300-bp and 195-bp products in the proband's parents (Fig. 1). Analysis of the exon 54–57 RT-PCR products by SSCP also demonstrated differences in the patterns of migration (data not shown). DNA sequence analysis of these products revealed that the smaller RT-PCR product lacked the nucleotide sequence encoding exon 55 of nebulin (GenBank accession no. NT_005403).

DNA sequencing of PCR-amplified regions of genomic DNA revealed that there was a 2,502-bp deletion in the NEB gene of the proband and that it included 2,025 bp at the 3′ end of intron 54, the 105 bp that encoded exon 55, and the 5′-most 372 bp of intron 55 (Fig. 2A). The deletion was designated R2478_D2512del to reflect the number and position of the 35 amino acids that the mutant allele fails to encode from exon 55. To assay for the mutant allele, PCR amplification was performed with a set of three primers designed to differentiate between the normal and mutated sequences of the NEB gene (Fig. 2B). The intron 54/intron 55 primers generated a 360-bp product from the mutant allele, and the exon 55/intron 55 primers generated a 672-bp product from the normal allele (a third product of 2,862 bp was also generated from the normal allele from the intron 54/intron 55 primers, but the short extension time of the PCR protocol favored amplification of the two smaller products). This analysis revealed that the DNA from the parents of all of the probands yielded both the 672-bp and 360-bp PCR products, that the DNA from the probands yielded only the smaller product, and that the DNA from an unrelated individual yielded only the larger PCR product (Fig. 3). Using this PCR screening methodology, we analyzed the carrier frequency of the R2478_D2512del mutation in DNA purified from 4090 anonymous individuals of Ashkenazi Jewish descent. In this sample, 38 individuals were found to carry this mutation, demonstrating a carrier frequency of approximately one in 108.

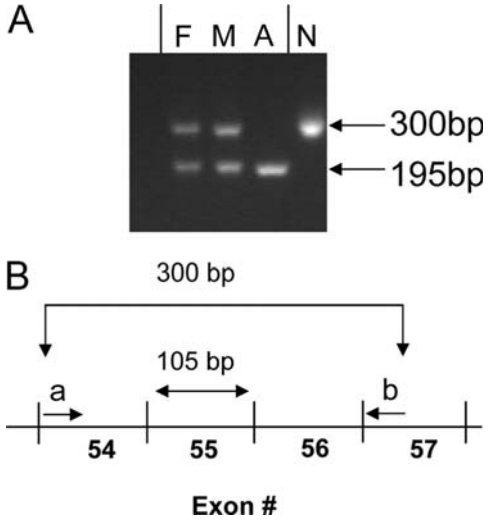

Fig. 1A, B **A** RNA extracted from whole blood was subjected to RT-PCR and run on a 2% agarose gel. Results presented are from one family consisting of the father (*F*), mother (*M*), and affected (*A*) child, and an unrelated non-carrier (*N*) of the mutation. **B** Depiction of the 300-bp (normal) and 195-bp (mutant) transcripts generated by RT-PCR. Primers in exon 54 (*primer a*) and exon 57 (*primer b*) generate a 300-bp RT-PCR product from the normal nebulin transcript and a 195-bp RT-PCR product (minus exon 55) from the mutant transcript

Discussion

Nemaline myopathy has been demonstrated to be the result of mutations in at least five different genes and at many different loci within these genes. Study of the NEB gene in individuals with autosomal recessive NM has revealed the presence of numerous disease-causing mutations. The mutations described to date include small deletions/insertions, nonsense and missense mutations, and splice site mutations, resulting in frameshifts, premature stop codons, amino acid substitutions, and abnormal splicing, respectively (Pelin et al. 1999, 2002; Wallgren-Pettersson and Laing 2003).

The NEB gene produces alternatively spliced RNAs encoding as many as 187 exons (Pelin et al.1999). The nebulin protein, which has been reported to range in size from 600 to 800 kD, is filamentous and comprises 3%–4% of total myofibrilla protein. A single nebulin molecule associates, along the entire length of the thin filament, with the C terminus anchored in the Z-disc and the N-terminus at the pointed end of the thin filament. The correlated size of nebulin with the length of the thin filaments suggests that nebulin acts as a molecular "ruler" that specifies the length of the thin filaments (Kruger et al. 1991; Labeit et al. 1991; Wright et al. 1993; Wang et al. 1996; Moncman and Wang 2000). Sequencing of human NEB cDNAs has demonstrated that the encoded protein contains approximately 185 tandem repeats of approximately 35 amino acid modules. These modules can be classified into seven types, and one of each type forms a seven-module set, yielding approximately 20 super repeats (Labeit and Kolmerer 1995; Pfuhl et al. 1996; Wang et al. 1996).

With an open reading frame of 20.8 kb, the large sizes of the NEB gene and transcript have greatly hindered the identification of NM-causative mutations in NEB. To facilitate the identification of such mutations in NM patients, some workers have begun to use antibodies generated against different regions of nebulin to characterize this protein. Nebulin molecules lacking certain epitopes have been detected in some patients with NM (Pelin et al. 1999; Gurgel-Giannetti et al. 2001, 2002; Sewry et al. 2001).

The identification of five families of Ashkenazi Jewish descent having one or more children with the typical form of NM prompted an investigation of the genetic cause of this disease. As haplotype analysis revealed that all of the

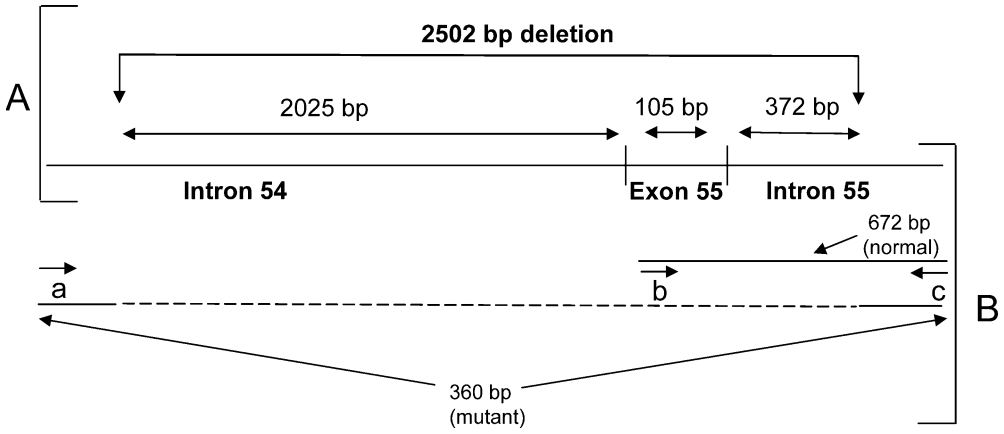

Fig. 2A, B **A** The 2,502-bp deletion. **B** Assay, by PCR amplification, of the mutant and normal alleles. The 360-bp product of the mutant allele is derived from intron 54/55 primers (*primer a*, *primer c*) and the 672-bp product of the normal allele is derived from exon 55/intron 55 primers (*primer b*, *primer c*). *Dashed line* The 2,502 bp missing from the mutant allele. DNA segments are not drawn to scale

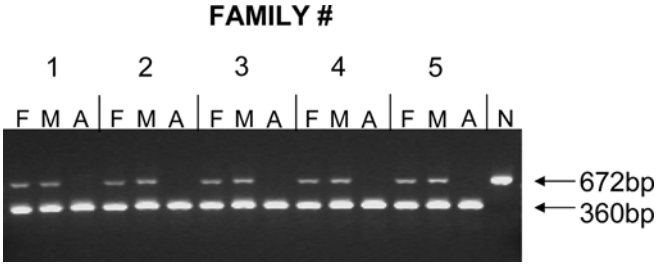

Fig. 3 PCR detection of the R2478_D2512del allele in five families. PCR was performed on the DNA from the five probands (*A* affected child), their parents (*F* father, *M* mother), and an unrelated non-carrier individual (*N*), and the products generated were run on a 2% agarose gel. The 672-bp product represents the normal allele and the 360-bp product, the mutant allele

NM-affected individuals were homozygous for two microsatellite markers located on either side of the nebulin-encoding gene, we examined the NEB gene for the causative mutation. Using RT-PCR analysis with primers specific for the nebulin transcript, we determined that the nebulin-encoding mRNA from the NM-affected individuals lacked the sequence encoded by exon 55 of the NEB gene. DNA sequence analysis of the genomic DNA of these individuals revealed a deletion of a 2,502-bp region that included parts of introns 54 and 55 and the entire exon 55 sequence. Parents of those with NM were found to be heterozygous for this mutation, and healthy siblings of the probands were either heterozygous for the mutated allele or homozygous for the normal allele.

The detection of this deletion in the nebulin-encoding gene represents the first report of a large NM-causing deletion in this gene. The absence of exon 55 in the nebulin transcript does not generate a frameshift, and the transcript is predicted to encode a protein that is 35 amino acids shorter than the normal gene product. Two of the encoded 35 amino acid modules of nebulin are interrupted by the deletion of exon 55, resulting in the disruption of the seven-module set of super repeat number nine (Labeit and Kolmerer 1995; Pfuhl et al. 1996). Since several studies have suggested that each nebulin module interacts with an actin module and that each super repeat interacts with a regulatory unit of the thin filament (Labeit and Kolmerer 1995; Labeit et al. 1991; Wang et al. 1996), the deletion of exon 55 of nebulin could affect both the length and function of thin filaments. It is interesting to note that exon 55 starts with the last four amino acids (RLYR) of a SXXXY(K/R) hexapeptide characteristic of each module (Labeit et al. 1991) and ends with the first two amino acids (SD) of a second hexapeptide. The finding that exon 55 encodes 35 amino acids and that the splicing of exon 54 to exon 56 results in the coding of a reconstituted hexapeptide (SDKLYR), suggests that, functionally, a single module may be missing, despite the interruption of two. When the amino acid sequences flanking exon 55 are subjected to secondary structure analysis, in the context of the surrounding amino acids, the probability of nearby amino acid sequences forming alpha helical structures decreases from a high probability (>80%) to a low probability (<40%) when exon 55 amino acids are removed (analysis by the Protein Sequence Analysis server, BioMolecular Engineering Research Center, Boston University: http://bmerc-www.bu.edu/; Stultz et al. 1993, 1997; White et al. 1994). The effect of these theoretical changes in secondary structure is not known, since the impact of alpha helicity on the binding of nebulin to thin filaments is not clear (Shih et al. 1997).

PCR-based screening of 4,090 unrelated individuals of Ashkenazi Jewish descent reveals that the R2478_D2512del mutation is present in one in 108 individuals or that the carrier frequency is 0.0093. The gene frequency for the R2478_D2512del mutation is comparable to that reported for Bloom syndrome (Li et al. 1998; Roa et al. 1999) and mucolipidosis type-IV-causing mutations (Bargal et al. 2001; Edelmann et al. 2002). Assuming that R2478_2512del is the primary, or only, causative mutation of NM in the Ashkenazi Jewish population, the noted carrier frequency predicts an estimated incidence of approximately two per 100,000 live births, which is similar to the rate at which NM is predicted to occur in the general population (Wallgren-Pettersson 1990).

The Ashkenazi Jewish population is at increased risk for several recessively inherited disorders (Tay-Sachs disease, cystic fibrosis, Canavan disease, Gaucher disease, familial dysautonomia, Niemann-Pick disease, mucolipidosis type IV, Fanconi anemia, and Bloom syndrome). The relatively homogeneous nature of this population and the often limited number of causative mutations in the population

have facilitated the identification of these mutations and the development of successful carrier screening programs (Kaback 2000; Ekstein and Katzenstein 2001; Sutton 2002).

The identification of several families of Ashkenazi Jewish descent with children who have NM, the observed carrier frequency of 0.0093, and the high detectability of carriers by testing for a single mutation make carrier testing for this mutation in the Ashkenazi Jewish population valuable and feasible.

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