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Clinical and genetic heterogeneity in autosomal recessive nemaline myopathy

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Abstract

Autosomal recessive nemaline (rod) myopathy is clinically and genetically heterogeneous. A clinically distinct, typical form, with onset in infancy and a non-progressive or slowly progressive course, has been assigned to a region on chromosome 2q22 harbouring the nebulin gene. Mutations have now been found in this gene, confirming its causative role. The gene for slow tropomyosin *TPM3* on chromosome 1q21, previously found to cause a dominantly inherited form, has recently been found to be homozygously mutated in one severe consanguineous case. Here we wished to determine the degree of genetic homogeneity or heterogeneity of autosomal recessive nemaline myopathy by linkage analysis of 45 families from 10 countries. Forty-one of the families showed linkage results compatible with linkage to markers in the nebulin region, the highest combined lod scores at zero recombination being 14.13 for the marker D2S2236. We found no indication of genetic heterogeneity for the typical form of nemaline myopathy. In four families with more severe forms of nemaline myopathy, however, linkage to both the nebulin and the TPM3 locus was excluded. Our results indicate that at least three genetic loci exist for autosomal recessive nemaline myopathy. Studies of additional families are needed to localise the as yet unknown causative genes, and to fully elucidate genotype-phenotype correlations.

Keywords: Nemaline myopathy; Nemaline (rod) myopathy; Congenital myopathy; Autosomal recessive; Genetic heterogeneity; Clinical forms; Genetic loci; Locus heterogeneity

1. Introduction

In familial cases of nemaline myopathy, the mode of inheritance is commonly autosomal recessive, although a few instances of autosomal dominant inheritance have been documented, including one extended family with male-to-male transmission [1,2]. No exact figures are available regarding the proportions of familial versus sporadic cases, but a rough approximation, based on the 150 families currently represented in the ENMC International database would be: familial cases 37% (likely autosomal recessive 24%, likely autosomal dominant 13%) and sporadic cases 63%. It is as yet unknown what proportion of the sporadic cases is due to autosomal recessive inheritance, and how

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Table 1
The typical form of nemaline myopathy

Inclusion criteria
Onset in infancy
Weakness especially pronounced in the facial, bulbar and respiratory muscles, and in the neck flexors
Proximal > distal weakness initially
Milestones delayed, but usually reached
Later distal involvement
Slowly progressive or non-progressive course
Exclusion criteria
At birth
No spontaneous movements
No spontaneous respiration
Contractures
Fractures
Adult or late childhood onset
Associated features:
Cardiomyopathy
Ophthalmoplegia
Unusual distribution of weakness
Intranuclear nemaline bodies

many might be due to new dominant mutations [3]. The cardinal features of all nemaline myopathies are muscle weakness and the presence of nemaline (rod) bodies in the muscle fibres [4–6]. The nemaline or rod bodies are derived from the Z disc of striated muscle and composed of Z-disc proteins [7–9].

Clinically, attempts have been made to classify the nemaline myopathies [10–13]. It appears that the disease spectrum forms a continuum, from prenatal, very severe forms through the typical congenital, usually slowly progressive form (Tables 1 and 2) to late-onset forms, and that any delineations will inevitably be more or less arbitrary and debatable [3,6]. In defining the categories presented in this paper, we have attempted to avoid at least the most obvious pitfalls and circular arguments inherent in this discourse.

Table 2
Severe nemaline myopathy

Inclusion criteria
Features
No spontaneous movements neonatally
No spontaneous respiration neonatally
Contractures at birth
Fractures at birth
Unable to achieve respiratory independence
Unable to achieve sitting
Unable to achieve walking
Exclusion criteria
Associated features:
Cardiomyopathy
Ophthalmoplegia
Unusual distribution of weakness
Intranuclear nemaline bodies

One of those would be defining any case of nemaline myopathy where the patient has deceased in infancy as being necessarily severe; survival in infancy will depend on the treatment provided [14] and there are several reports of patients with mild forms of nemaline myopathy suddenly going into respiratory failure because of undiagnosed insidious hypoventilation [15–19]. Adult-onset forms, with no dysmorphic features secondary to muscle weakness, and often with a progressive course, may not be genetically caused and are beyond the scope of this paper.

To date, two different genetic loci have been implicated in autosomal recessive nemaline myopathy. The typical form (Table 1) showed linkage to a region on chromosome 2q22 harbouring the nebulin gene [20,21], and, recently, mutations have been found in five unrelated families, confirming that nebulin is the causative gene [22]. At another locus, on chromosome 1q21, homozygosity for a nonsense mutation was found in the alpha-tropomyosin gene *TPM3* in one patient with consanguineous parents [23]. The family, however, has been lost to follow-up, and the parents, healthy by history, have therefore not been examined clinically nor investigated for the mutation. Thus, although autosomal recessive inheritance appears likely, homozygosity for a dominant mutation cannot be excluded. This boy had an intermediate form of nemaline myopathy in that he was said to have had normal muscle strength at birth, but was still unable to sit when he died of pneumonia at the age of 21 months. His muscle biopsy showed slight predominance of type 2 fibres, which were larger than type 1 fibres, and nemaline bodies present in smaller type 1 fibres only. The histological findings are comparable to those of the Australian family with a dominantly inherited mutation in the *TPM3* gene [1,2].

In an effort to determine the degree of genetic homogeneity or heterogeneity of autosomal recessive nemaline myopathy we present a linkage study of 45 families from different parts of the world. Our results show that there is at least a third locus for autosomal recessive nemaline myopathy, reinforcing the view that the nemaline myopathies are genetically heterogeneous.

2. Patients and clinical definitions

Linkage studies were performed in 45 families from 10 different countries. Twenty-five families were multiplex families where unaffected parents had two or more children affected by nemaline myopathy. The remaining 20 families included sporadic cases with the affected child having at least one healthy sibling, or, in three instances, consanguineous parents, the family structure thus permitting linkage analysis.

There were 26 families with probands who met our criteria of the typical form of autosomal recessive nemaline myopathy (Table 1). Sixteen of these were multiplex.

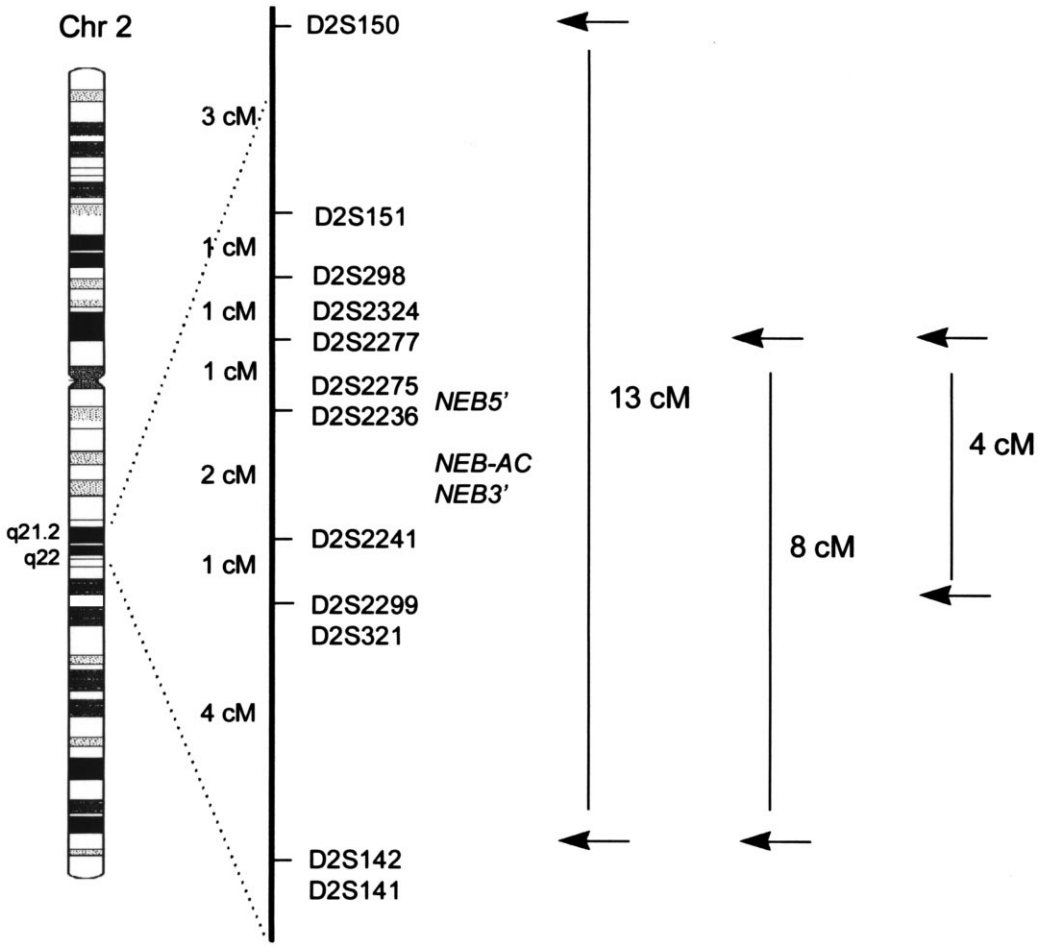

Fig. 1. The linkage region on chromosome 2q21.1-q22 for the typical form of autosomal recessive nemaline myopathy. Linkage to the 13 cM region between markers D2S150 and D2S142 was originally reported in 7 multiplex families [20]. Recombinations in four new multiplex families have now reduced the region to 4 cM. The nebulin gene (*NEB*) is located within the linkage region [21], and this gene has recently been found to be mutated in six cases of autosomal recessive nemaline myopathy [22].

Among the 10 families of typical sporadic cases there were two in which the parents were consanguineous.

The remaining 19 families showed other types of nemaline myopathy; in 11 the patients fulfilled the criteria in Table 2 and were classified as having severe nemaline myopathy. Four cases with unusual associated features (Tables 1 and 2, exclusion criteria) were assigned to the category of 'other forms', and four were not classified because the available clinical data were insufficient. Among these 19 families, there were nine with affected sib pairs, and in one of these families, the parents were consanguineous. A further consanguineous couple had a single affected child, and in the remaining nine families there were singleton patients with healthy sibs.

2.1. The typical form of autosomal recessive nemaline myopathy

Defined here as follows [3,17,24,25] (Table 1): onset is in infancy, the infant often being floppy at birth, with feeding difficulties and insufficient respiration. The muscle weakness is generalised, but most pronounced in the facial, neck flexor, bulbar, and respiratory muscles. The proximal muscles of the limbs are initially weaker than the distal ones, but later, there is usually also a distal involvement, the dorsiflexors of the feet being especially severely affected. The extra-ocular muscles are spared.

The facies is myopathic, the palate high-arched, and the gag reflex is typically absent. The gait is waddling and the build is usually slender. The spine is hyperlordotic, or sometimes rigid, and scoliosis is common, with onset usually in the prepubertal period of rapid growth. Tendon reflexes are weak or absent. Gross motor activity is slow whereas fine motor activity is normal. Chest deformities are common, even in small children, and contractures and deformities of the joints often develop over time. Intelligence is normal, and a small series showed a skew towards higher levels [17]. Cardiac contractility is usually normal [3,26] and there is no involvement of smooth muscle. Nerve conduction velocities are normal, and the electromyographic findings usually progress from normal to 'myopathic' in proximal muscles, and from normal over 'myopathic' to 'neurogenic' in distal

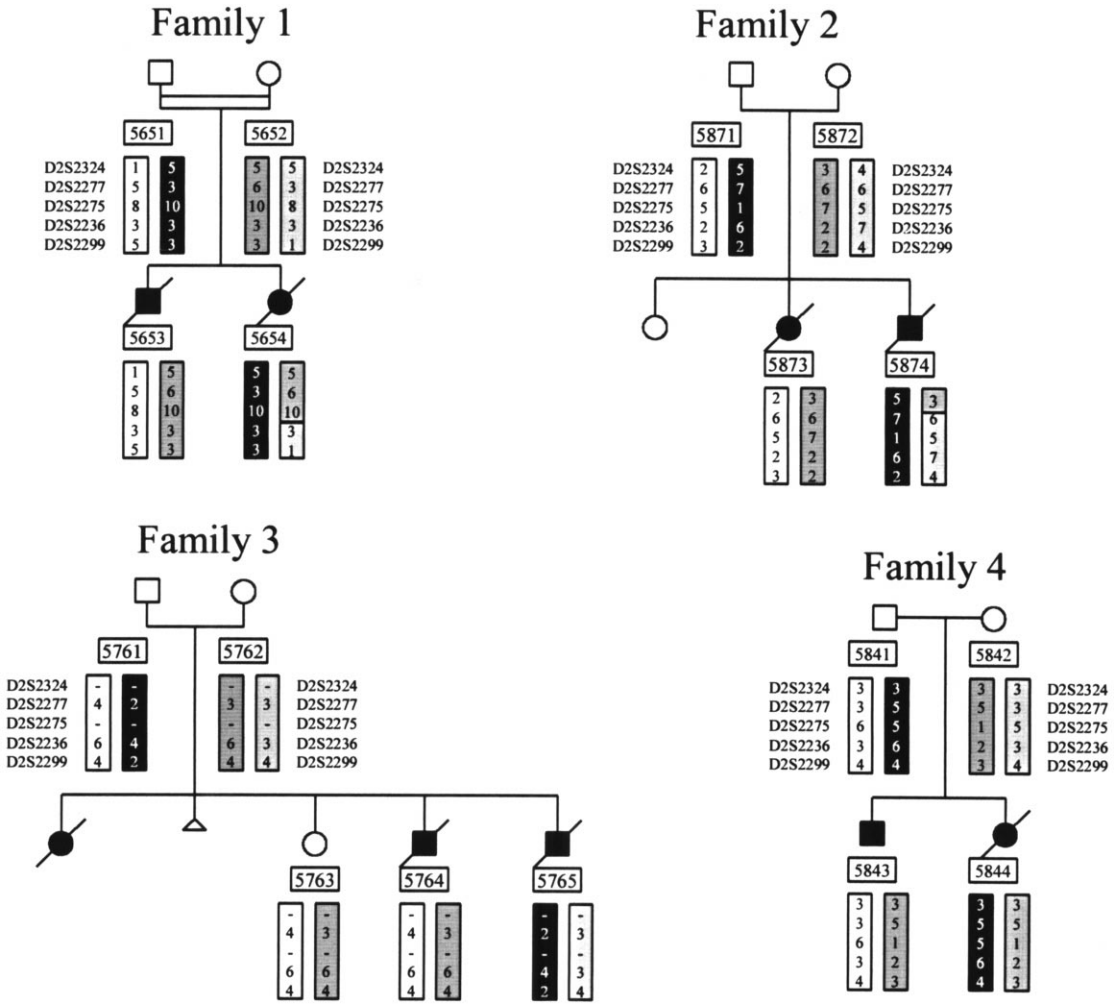

Fig. 2. Pedigrees and haplotypes for markers in the 2q22 linkage region for four families with sib pairs affected by severe forms of nemaline myopathy (described in Section 2). The haplotypes are shown in shades from white to black to indicate which haplotype in each case was inherited from the mother and which one from the father. The affected sibs in each family have different haplotypes at this locus. In all four families, this excludes linkage to the 2q22 locus.

muscles, reflecting segmental degeneration and reinnervation of muscle fibres [25]. Ultrasonography of muscles shows high echogenicity, computed tomography shows low density of muscles with preservation of volume, and MRI commonly reveals fatty infiltration over time [24]. Serum levels of creatine kinase are normal or slightly elevated.

Muscle biopsy shows the presence of nemaline bodies and often both predominance of type 1 fibres and deficiency of one or both subtypes of type 2 fibres [27,28]. On follow-up, unspecific myopathic changes progress slowly over the years. There are no dystrophic changes, nor is there any inflammatory component. Patients who remain ambulant often have hypertrophy of a population of muscle fibres [28].

Affected children usually survive infancy if actively treated, and achieve motor milestones, albeit later than normal. The chief clinical concerns are the respiratory function, the swallowing difficulties with associated risk of aspiration, and the possible development of scoliosis [3,5]. Many patients remain ambulant as adults.

2.2. Severe nemaline myopathy

Severe nemaline myopathy constitutes a category of patients who at birth had one or more of the following clinical features: absence of spontaneous movements or of spontaneous respiration, or presence of contractures or fractures. The prognosis is often, but not uniformly, poor [14]. Also included in this category were patients who breathed at birth but who in early childhood became permanently ventilator-dependent, and those unable to achieve sitting or walking (Table 2).

Among the 11 families classified as showing severe nemaline myopathy, linkage to the 2q locus was excluded in four. In family 1 (Figs. 2 and 3), consanguineous parents had two children with contractures of several joints and lack of antigravity movements at birth. The children died at 5

Table 3
Disease severity and linkage to the nebulin locus, shown as maximum pairwise lod scores for the microsatellite markers D2S2275 and D2S2236 (closest to *NEB*), in 45 families with nemaline myopathy of disease categories described in detail under Section 2

Family no.	Case status	Category of nemaline myopathy	Lod score D2S2275	D2S2236
1	Affected sib pair	Severe form	$-\infty$	$-\infty$
2	Affected sib pair	Severe form	$-\infty$	$-\infty$
3	Affected sib pair	Severe form	Not done	$-\infty$
4	Affected sib pair	Severe form	$-\infty$	$-\infty$
5	Affected sib pair	Typical form	0.85	0.25
6	Affected sib pair	Typical form	0.12	0.73
7	Affected sib pair	Typical form	0.43	0.12
8	Affected sib pair	Typical form	0.60	0.52
9	Affected sib pair	Typical form	0.85	0.98
10	Affected sib pair	Typical form	0.60	0.60
11	Affected sib pair	Typical form	0.43	0.73
12	Affected sib pair	Typical form	0.25	0.85
13	Three sibs affected	Typical form	1.33	1.33
14	Affected sib pair	Typical form	0.73	0.73
15	Affected sib pair	Typical form	0.30	0.60
16	Affected sib pair	Typical form	0.30	0.30
17	Affected sib pair	Typical form	0.73	0.00
18	Affected sib pair	Typical form	0.85	0.85
19	Affected sib pair	Typical form	0.00	0.98
20	Affected sib pair	Typical form	−0.07	0.37
21	Sporadic case	Typical form	0.12	0.00
22	Sporadic case	Typical form	0.00	0.12
23	Sporadic case	Typical form	0.12	0.25
24	Sporadic case	Typical form	0.12	0.12
25	Sporadic case	Typical form	0.12	0.00
26	Sporadic case	Severe form	0.12	−0.14
27	Sporadic case	Typical form	−0.18	0.12
28	Sporadic case	Not classified	0.00	0.12
29	Affected sib pair	Severe form	0.73	0.12
30	Sporadic case	Typical form	0.00	0.00
31	Sporadic case	Severe form	0.00	0.12
32	Sporadic case	Not classified	−0.18	0.12
33	Sporadic case	Typical form	0.25	0.25
34	Sporadic case	Other form	0.12	0.00
35	Affected sib pair	Severe form	1.23	1.23
36	Sporadic case	Other form	0.12	0.12
37	Affected sib pair	Other form	0.30	0.30
38	Affected sib pair	Severe form	0.73	0.12
39	Sporadic case	Severe form	0.12	0.12
40	Sporadic case	Typical	0.12	0.12
41	Sporadic case	Typical	0.12	0.12
42	Sporadic case	Not classified	0.25	−0.05
43	Affected sib pair	Severe	0.73	0.73
44	Sporadic case	Other form	0.12	0.00
45	Sporadic case	Not classified	0.12	0.12

weeks and 10 months, respectively [29]. In family 2, the clinical picture was similar, and the affected sibs died at ages of 1 and 4 days, respectively. Two of the three affected children of family 3, had severe hypotonia, clubfeet and respiratory insufficiency, and died of respiratory failure within a month of birth. The first child of this family, from whom no sample was available for study, had no contractures, but was ventilated from the age of 2 weeks and died at 18 months. In family 4, one of the affected, severely hypotonic sibs was still alive at age 10 years but was unable to sit. The other child died at the age of 5 years. In the remaining seven families in the category of severe nemaline myopathy, linkage to the 2q22 locus could not be excluded. The clinical picture in these families was heterogeneous, but fulfilled the criteria in Table 2.

2.3. Other forms of nemaline myopathy

Other forms of nemaline myopathy are defined as those with one or more aberrant features rarely associated with nemaline myopathy, such as ophthalmoplegia, cardiomyo-

Table 4
Pairwise combined lod scores of 16 multiplex families with the typical form of nemaline myopathy for markers in the nebulin region on chromosome 2q22

Locus	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D2S2324	−∞	3.93	4.55	4.15	2.84	1.43	0.38
D2S2277	−∞	2.13	4.03	4.10	3.04	1.62	0.46
D2S2275	8.30	8.08	7.20	6.07	3.89	1.92	0.54
D2S2236	9.94	9.70	8.70	7.39	4.87	2.48	0.67
D2S2299	−∞	6.39	6.26	5.48	3.67	1.86	0.51
D2S321	3.41	3.33	2.98	2.54	1.66	0.84	0.24

pathy or intranuclear nemaline bodies (Tables 1 and 2, exclusion criteria).

3. Methods

The following polymorphic microsatellite markers were used in the linkage analyses: D2S2324, D2S2277, D2S2275, D2S2236, D2S2299, D2S321 for the nebulin locus, D2S300, D2S384, TTN-AC and D2S364 for the titin locus, D15S987, TPM1STR and D15S1018 for the tropomyosin 1 locus, D9S163, 99L21sp6CA for the tropomyosin 2 locus, D1S252, D1S305, D1S303 and D1S194 for the tropomyosin 3 locus, D19S221, D19S226, D19S411 and D19S410 for the tropomyosin 4 locus, ACTN2-AC for the α-actinin 2 locus, and D11S1965, D11S913, D11S916 and D11S906 for the α-actinin 3 locus. TTN-AC (Labeit et al., unpublished data), TPM1STR [30], and ACTN2-AC (AC M86804) [31] are polymorphic intragenic microsatellite markers, 99L21sp6CA is a polymorphic AC-repeat 0.5 cM from D9S163 [32]. All the other markers were from Généthon [33].

Polyacrylamide chain reaction conditions were as described previously [21]. The PCR products were run on 5–6% polyacrylamide gels, and the alleles were visualised by silver staining [34]. The alleles were numbered consecutively, '1' being the largest allele.

Two-point LOD-score calculations were performed by use of the MLINK option of the LINKAGE programme package [35–37].

Where linkage analysis at the nebulin locus showed exclusion, linkage studies were extended to other potential candidate loci, i.e. those of the sarcomeric proteins titin, the tropomyosins 1–4 and the alpha-actinins 2 and 3. Mutations in the *TPM3* gene were excluded by SSCP screening in all families, and in family 3, the *TPM1* gene was sequenced in an unsuccessful attempt to find a mutation.

Table 5
Pairwise combined lod scores of 41 families for markers in the nebulin region on chromosome 2q22

Locus	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D2S2324	−∞	6.61	6.89	6.09	4.01	2.03	0.56
D2S2277	−∞	5.04	6.55	6.16	4.28	2.22	0.63
D2S2275	13.65	13.28	11.71	9.79	6.16	3.04	0.83
D2S2236	14.13	13.77	12.27	10.39	6.72	3.42	0.96
D2S2299	−∞	10.78	10.02	8.54	5.47	2.76	0.77
D2S321	4.23	4.12	3.68	3.13	2.03	1.05	0.29

4. Results

The maximum lod scores of individual families at the 2q22 locus are presented in Table 3. Although none of the individual families had a family structure providing lod scores high enough for significant linkage, families with the typical form all showed results compatible with linkage to the nebulin locus on chromosome 2q22. For all the 16 multiplex families with this form (in which the mode of inheritance is most likely to be autosomal recessive), the marker D2S2236 gave the highest combined two-point lod score of 9.94 at zero recombination (Table 4). Combined lod scores of all the 41 families with linkage results compatible with linkage to the nebulin locus, 26 of which showed the typical form, seven severe forms and four other forms, and four of which could not be classified, showed a maximum of 14.13 for the marker D2S2236 at zero recombination (Table 5). Through the detection of recombinations in two multiplex families with the typical form, the linkage region was narrowed down to 4 cM (Fig. 1). Lod score tables on the individual families are available on request.

Linkage to the nebulin locus was excluded in four families with severe forms of nemaline myopathy (Fig. 2). In these four families, linkage to the *TPM3* locus was also excluded (Table 6). Linkage analysis with regard to six other potential candidate loci did not yield any suggestion of the families showing linkage to the same locus. In one of the four families, family 3, linkage to all but the *ACTN2* and the *TPM1* loci was excluded, but *ACTN2* gave negative lod scores and sequencing of the *TPM1* gene in this family disclosed no mutations. In two further families, linkage to all but two of the loci was excluded, and in the fourth, linkage analysis gave slightly positive lod scores at four different loci (Table 6, Fig. 3).

5. Discussion

This study provides further evidence for autosomal recessive nemaline myopathy being clinically and genetically heterogeneous.

On clinical grounds, we defined a typical form of autosomal recessive nemaline myopathy, a category of severe forms, and a third category of 'other forms', comprising those with unusual features differentiating them from the other two categories. All 26 families with the typical form and 15 families with severe or other forms showed linkage results compatible with linkage to the nebulin locus, whereas four families with severe forms showed exclusion of linkage to this locus.

The typical form was shown to be tightly linked to a

Table 6
Pairwise lod scores at zero recombination for eight candidate loci in four families with severe forms of nemaline myopathy

Region q14	2q21.1–q22 Nebulin	2q24.3 Titin	15q22 TPM1	9p13 TPM2	1q22–q23 TPM3	19p13 TPM4	1q42–q43 ACTN2	11q13–q14 ACTN3
Family								
1	−∞	0.60	−∞	−∞	−∞	−∞	0.60	−∞
2	−∞	−∞	0.60	−∞	−∞	0.60	−∞	−∞
3	−∞	−∞	0.43	−∞	−∞	−∞	−0.18	−∞
4	−∞	−∞	−∞	0.00	−∞	0.60	0.60	0.30

region on chromosome 2q22, now restricted to 4 cm, harbouring the nebulin gene, in which mutations have recently been found [22]. In the ENMC International Database, currently containing entries on some 180 patients, the majority of cases have the typical form. The proportions may, however, be biased by fatal neonatal cases and very mild late-onset cases being underdiagnosed.

Future studies will show whether all cases of the typical form are caused by mutations in the nebulin gene, and whether any of the clinically different forms will be shown to be due to pathogenetically different mutations in this gene. In all families in which nebulin mutations have been found to date, the mutations have been different [22], and further mutation detection in this very large gene will require great effort.

The clinical categories of 'severe nemaline myopathy' and 'other forms of nemaline myopathy' were defined on the basis of two hypotheses, related to possible pathogenetic mechanisms. The first one is that the severe end of the spectrum might represent one or more distinct entities, differing molecularly, genetically and pathogenetically from the typical form. This refers to cases where the foetus or the new-born infant has no spontaneous movements at all, in many including no respiratory effort. In some of these cases, the muscle biopsy has shown lack of normal muscle structure [38], and some have been arthrogrypotic [29]. Our results excluding linkage to the nebulin locus in four informative families with severe nemaline myopathy support this hypothesis. The lack of exclusion in the other seven families with severe nemaline myopathy do not argue against it, especially in view of the small family sizes.

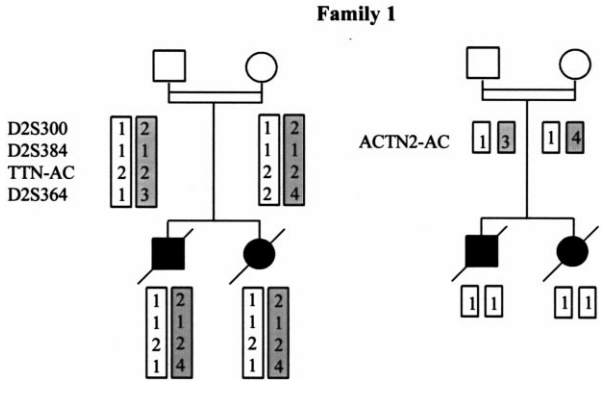

Fig. 3. Linkage results at two loci in family 1. Haplotypes for markers in the titin region on chromosome 2q, and alleles for a marker, ACTN2-AC, in the alpha-actinin region on chromosome 1q. The haplotypes are shown in white and shades of grey, respectively, to indicate which haplotype was inherited from the mother and which one from the father.

The other hypothesis relates to unusual associated features perhaps indicating different causative mechanisms. An example of this would be patients with intranuclear nemaline bodies. The families included in this category were not informative enough for this hypothesis to be tested by linkage studies.

In both categories, informative families are currently too few and clinically too heterogeneous for a genome-wide search for linkage to be likely to succeed.

For the families in the category of severe nemaline myopathy in which linkage to the nebulin locus was excluded, linkage analysis extended to seven other candidate loci, i.e. those for titin, the tropomyosins, and two of the alpha-actinin genes, revealed no common locus of linkage. In particular, the families showed exclusion of linkage to the *TPM3* locus also, where one patient, clinically fitting into our category of severe nemaline myopathy, was previously reported to be homozygous for a nonsense mutation [23]. Due to small family size, exclusion was, however, tentative. The slightly positive lod scores in these families at various loci (Table 6) can also only be regarded as tentative. In family 3, showing results compatible with linkage to the tropomyosin locus on 15q22, mutations in the *TPM1* gene were sought but none were found. Further candidate loci and genes will be analysed as they become known.

6. Conclusion

Our results indicate that there are at least three genetic loci for autosomal recessive nemaline myopathy, and that locus heterogeneity explains at least some of the clinical heterogeneity in autosomal recessive nemaline myopathy. Studies of further families and genetic loci are needed to identify the remaining causative genes. Full analysis of the relationship between the clinical disease spectrum and the molecular genetic cause awaits further mutational results.

7. Note added in proof

Recently, mutations in a third gene have been found to cause nemaline myopathy and actin myopathy (Nowak et

al., Nature Genetics, accepted for publication). As some of the families described in the current paper show exclusion of linkage to this as well as the other two loci, it appears that there are at least four genes causing nemaline myopathy.

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