ORIGINAL ARTICLES

Integrated study of 100 patients with Xp21 linked muscular dystrophy using clinical, genetic, immunochemical, and histopathological data. Part 1. Trends across the clinical groups

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Abstract

This multidisciplinary study was undertaken to record the variation in gene and protein expression in a large cohort of patients with well defined clinical phenotypes. The patients, whose ages ranged from 4 years to 66 years, spanned a wide range of disease severity. They represented the first 100 patients who had been examined in Newcastle, had undergone a muscle biopsy, and provided a blood sample for DNA analysis. The study had three aims: to observe any trends in the analyses across the clinical groups, to correlate gene and protein expression in individual patients, and to use the data collected to assess the relative usefulness of different techniques in the diagnosis and prognosis of patients with Duchenne and Becker dystrophy (DMD/BMD). In part 1, we describe the clinical assessment of the patients and the trends that were observed across the cohort. The patients were divided into seven groups. Group 1 had severe DMD (n=21), group 2 had milder DMD (n=20), group 3 were intermediate D/BMD patients (n=9), group 4 had severe BMD (n=5), and group 5 were more typical BMD patients (n=31). Some patients were too young to be classified (n=7) and a group of all the female patients were also classified separately (n=7). The number of DMD and BMD patients was about equal, in accord with disease prevalence in the north of England, but an unusually high proportion were sporadic cases. Dystrophin labelling (performed with up to three antibodies) on both blots and sections increased gradually across the clinical groups. All histopathological indices, except the proportion of fat in biopsy sections, showed clear trends across the groups.

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Duchenne and Becker muscular dystrophy (DMD, BMD) are both caused by defects in a gene on the X chromosome located at Xp21.^{1,2} The defects cause abnormal expression of the

protein product, dystrophin, apparently in all patients.^{3,4} Most intragenic mutations are deletions, but duplications and point mutations have also been identified.⁵⁻⁷ The clinical spectrum of Xp21 linked muscular dystrophy varies from severe DMD, where the progressive loss of muscle function results in wheelchair dependency from about 7 years and death before 20 years, to mild BMD where a wheelchair may never be needed and a normal life span is frequently attained. In order to understand the pathogenesis of Duchenne and Becker dystrophy, the extent of variation in genotype and phenotype expression needs to be recorded. To this end we have undertaken a multidisciplinary investigation with the following three aims: to identify any patterns or trends in the analyses which would be shown by investigating a cohort of patients with a wide range of disease severity, to relate gene and protein defects in individual patients with well defined clinical phenotypes, and to identify which techniques are the most important for differential diagnosis and prognosis. The whole report has been divided into three sections, each addressing one of the aims defined above. In this first part we define the patient groups and report the pattern of results obtained across the clinical spectrum.

Materials and methods

PATIENT SELECTION

The 100 patients (93 male and seven female) included in this study were the first 100 patients with confirmed Xp21 linked muscular dystrophy who fulfilled the selection criteria of (1) having been examined at the Regional Neurosciences Centre of Newcastle General Hospital, (2) having undergone a muscle biopsy (which was stored in a liquid nitrogen archive), and (3) having provided a blood sample for lymphocyte DNA analysis. A further requirement was that, as far as possible, the patients were not related. Nevertheless, the 100 patients represent 98 family lines: also included were a nephew and uncle from one severe BMD family (patients F58 and F59 in the appendix) and a BMD father and his obligate carrier daughter (patients F67

and F94). The results from the genetic analysis were not known when the patient cohort was assembled but simple screening for dystrophin with a single antibody had been performed to confirm the initial clinical diagnosis.

CLINICAL ASSESSMENT

All patients in this study were examined by the same clinicians (DGM/KMDB). The following were recorded for each patient as appropriate: family history, age first walked, first symptoms, age first symptoms were observed, age at diagnosis, initial diagnosis, age at biopsy, age when ability to walk independently was lost, length of time in callipers (if used) before becoming wheelchair bound, length of time in wheelchair before death, age at death, IQ, age first words spoken, age first sentences constructed, achievements at school, further education, and employment undertaken. During the early years the times taken to run along a standard 11 m length of corridor, to climb six stairs, and to get up from the floor were recorded at every hospital visit. Measurements of forced vital capacity were also made at intervals following the loss of independent mobility. The disability of Becker patients was assessed according to the Cornelio scale.^{8,9} The strength of all the major muscle groups was tested and the results were related to age, a low score indicating little reduction in muscle function. Electrophysiological studies (EMG/ECG) had been performed on too few patients in this cohort for inclusion.

PATIENT CLASSIFICATION

The patients were basically classified according to the standard clinical criterion of the age at which independent mobility was lost and full length callipers or a wheelchair had to be used. Thus DMD patients lost the ability to walk independently by 12 years of age, intermediate D/BMD patients between 12 and 16 years, and BMD patients could walk independently until they were at least 16 years old. For the purposes of comparison in this investigation, however, the number of clinical groups was expanded. Among our entire population of DMD patients (not just those in this study) the average age of becoming wheelchair bound is just over 9 years. We therefore divided the DMD patients in this study into two groups: those in a wheelchair before 9 years (group 1, $n=21$) and those who lost mobility between 9.1 and 12 years of age (group 2, $n=20$). Patients with intermediate clinical symptoms (wheelchair bound 12.1 to 16 years) formed group 3 ($n=9$). The BMD patients were also divided into two groups: those in a wheelchair between 16 and 40 years (group 4, $n=5$) and those not in a wheelchair until past 40, if at all (group 5, $n=31$). Patients who were still ambulatory were assigned to clinical groups according to muscle function tests: group 4 patients corresponding to the 'severe Becker' group and group 5 to the 'typical Becker' group differentiated on the basis of Cornelio muscle function analysis described in detail by Bushby and Gardner-Medwin in 1993.⁹ The females were considered together as a separate

group (group F, $n=7$). One (patient S95 in the appendix) was the single affected member of a pair of monozygous twins.¹⁰

The current age range in our cohort of patients is from 4 to 66 years. Some of these patients could still walk independently. For the purposes of this study, most of these patients could be assigned to a clinical group according to their rate of disease progression. Detailed records are kept of every visit to the Regional Neurosciences Centre made by patients with muscular dystrophy so that a very large data base has been generated over the last 30 years. Thus it was possible to plot, for example, the time taken at different ages to run along an 11 m corridor or to get up from the floor for a very large number of patients in whom the whole clinical course had subsequently been recorded. By comparing the early data on patients whose clinical course was known with data collected on young patients who had not yet lost independent mobility, most young patients could be assigned to one of the five clinical groups. Nevertheless, patients under 6 years of age were still considered too young for assignment (group TY, $n=7$) and we readily acknowledge that it is not possible to predict a clinical course from early observations with complete accuracy. It must also be emphasised that patients with Xp21 linked muscular dystrophy present a continuous, though not evenly distributed, spectrum of disease severity and separation into five clinical groups was undertaken purely for comparative purposes in this study. The criteria used have no implications for classification for diagnostic purposes.

GENETIC ANALYSIS

Analysis of DNA isolated from lymphocytes and digested with the restriction enzyme *Hind*-III was performed using dystrophin cDNA probes 1-2a, 2b-3, 4-5a, 5b-7, 8, 9-10, and 11-14¹⁵ according to established techniques.^{6,11} In some instances PCR analysis was performed using multiplex primers.¹² The genetic analysis for 50 patients was performed in Leiden, The Netherlands. Deletions and duplications could be detected but not point mutations or other very small genetic rearrangements. X chromosome inactivation studies were undertaken for the female patients using differential methylation and fluorescent in situ hybridisation techniques which have been described in detail previously.^{10,13}

ANTIBODIES USED FOR PROTEIN ANALYSIS

Four monoclonal antibodies (MAbs) were used during this study: three to dystrophin and one to β -spectrin. The rod and C-terminal dystrophin MAbs detect epitopes on either side of the major deletion 'hotspot'. Generation and use of the rod domain MAb, Dy4/6D3 (DYS1), has been described previously.^{14,15} The immunogen was the 30 kDa fusion protein reported by Hoffman *et al*² and the epitope recognised is between amino acids 1181 and 1388 (exons 26 to 30 approximately). The C-terminal MAb, Dy8/6C5 (DYS2), was generated by immunisation with a synthetic

peptide consisting of the last 17 amino acids (3669–3685) of the human dystrophin sequence¹⁶ conjugated to keyhole limpet haemocyanin. The N-terminal MAb, Dy10/12B2 (DYS3), was generated by immunisation with a fusion protein containing cDNA Cf27,¹⁷ deletion analysis localised the epitope to the vicinity of exons 10 to 12, and subsequent epitope mapping with synthetic peptides (kit from Cambridge Research Biochemicals, UK) suggests that the antibody binds to sequences within amino acids 308 to 351 which span the junction of exons 9 and 10. This may be part of a hinge region joining the amino domain to the central rod domain¹⁸ and reactivity with a conformational epitope would explain why labelling with Dy10/12B2 is much weaker on blots but stronger on tissue sections than the other dystrophin MAbs. None of the dystrophin MAbs reacts with the chromosome 6 encoded dystrophin-like protein,⁹ also known as dystrophin related protein, DRP,²⁰ or utrophin.²¹ The β -spectrin MAb, RBC2/3D5 (SPEC1), was generated by immunisation with human red blood cell membrane ghosts.²² Spectrin is localised at the periphery of muscle fibres like dystrophin and labelling of this protein is thus an excellent control for muscle membrane integrity in fibres which appear negative for dystrophin labelling. All four antibodies (DYS1, 2, 3, and SPEC1) can be obtained from Novocastra Laboratories, 22 Claremont Place, Newcastle upon Tyne NE2 4AA, UK.

IMMUNOCYTOCHEMISTRY

Details about the site, size, and freezing of blocks of tissue from muscle biopsies have been described previously.¹⁵ The immunocytochemical analysis was performed on 6 μ m unfixed frozen tissue sections. The indirect horseradish peroxidase technique was used with visualisation of the label achieved by exposure to H_2O_2 /diaminobenzidine.¹⁵ All the biopsies were labelled with the rod MAb and in most cases serial sections were labelled with the C-terminus MAb. The amino-terminal MAb was used in a few cases when, for example, a patient had a deletion in the rod domain that removed the Dy4/6D3 binding site. The immunocytochemical labelling patterns were divided into eight categories according to the most prominent features observed: ICC 1 – no labelling on any fibres; ICC 2 – faint labelling on occasional fibres, rest negative; ICC 3 – clear labelling on a few fibres (generally < 1%), rest negative; ICC 4 – labelling of severely decreased intensity on up to 50% of fibres, a few may be more intensely labelled, rest negative; ICC 5 – labelling of severely decreased intensity on nearly all fibres; ICC 6 – labelling shows variation between and/or within fibres; ICC 7 – labelling is uniform but decreased in intensity; ICC 8 – labelling at near normal intensity (indistinguishable from controls). Figures illustrating labelling patterns like these may be found in previous papers.^{15,23}

WESTERN BLOTTING

Sample preparation, polyacrylamide gel electrophoresis (4 to 7% gradient resolving gel,

3% stacking gel), and Western blotting were performed as described previously¹⁴ as were the estimates of dystrophin size²⁴ and abundance.^{15,25} Dystrophin abundance was estimated by scanning densitometry of the myosin staining on post-blotted gels and the uppermost dystrophin band labelled in each blot lane. Thus, dystrophin abundance (expressed as a percentage of normal) could be adjusted for the amount of muscle protein (as opposed to fat and fibrous connective tissue) in the weight of sample homogenised. The principle of the densitometric analysis has been described in detail previously.²⁵ All biopsies were labelled with the rod MAb, most with the C-terminal MAb, and a few with the N-terminal MAb as in the immunocytochemical analysis.

HISTOPATHOLOGY

For histopathological examination frozen tissue sections 10 μ m thick were cut, post-fixed in formol calcium, and stained with haematoxylin and eosin. The percentage area of each biopsy occupied by fat and fibrous connective tissue was measured using a conventional stereological (point counting) technique.²⁶ Photomicrographs of tissue sections printed at a constant $\times 150$ magnification were used. Four areas per biopsy were analysed comprising a total of 5 mm² except when the size of the biopsy did not permit this. Control data derived from a series of patients with no histological evidence of neuromuscular disease showed that the normal upper limits of fibrous connective tissue and of fat in skeletal muscle are about 12% and 0.5% respectively. The incidence of features which indicate acute histopathological change (hyaline fibres, necrotic fibres, regenerated fibres), and those which indicate chronic change (ring fibres and fibre splitting) were estimated semi-quantitatively. Four categories of severity were defined for each histological feature: 0 = nil, 1 = < 2%, 2 = 2–5%, and 3 = > 5%. The incidence of groups of fibres showing degeneration and regeneration was also assessed. To circumvent the problem of age related changes in the absolute size of muscle fibres, the range of fibre diameters was expressed as n-fold variation (for example, 5-fold, 10-fold). The normal upper limit of fibre size variation is 3-fold. The histopathological and immunocytochemical analyses were performed on all the biopsies by the same person (MAJ).

STATISTICAL ANALYSIS

Each person responsible for an aspect of this study completed collection of their data without knowledge of all the results from other participants. The data were entered into a database programme and analysed only at the completion of the study. The values for dystrophin size and abundance given in the appendix are the final numbers that were arrived at after the calculations described above and have not been simplified. Tests used were the Student *t* test, paired *t* test, χ^2 test, one way analysis of variance (ANOVA) followed by multiple comparison tests to identify individual differences, and regression analysis. A *p* value of greater than 0.05 was considered to be 'not significant'.

Results

GENETIC ANALYSIS

The 100 patients in this study were from 98 family lines. Of 92 unrelated male patients, 13 (14%) had affected relatives in a previous generation, 12 (13%) had affected brothers only, and 67 (73%) were sporadic cases (patient numbers prefixed F, B, or S respectively in the appendix). Among the women, six were isolated cases and one was the daughter of a BMD patient who was also included in this study.

Table 1 summarises the gene mutations (deletions or duplications) found in each of the clinical groups. There were some subjects within every clinical group for whom no mutation could be found by Southern blot analysis. Deletions/duplications of the Xp21 gene were detected in 81.5% (75/92) of all male patients, 80.6% (54/67) occurring in isolated cases and 84% (21/25) in families with more than one affected member (data pooled for previous affected generations ($n=13$) or brother pairs ($n=12$) since, for these very small numbers, no statistical difference was detected). The proportion of sporadic cases versus multiple member families with such mutations was not significant. The ratio of mutations in the proximal and distal 'hotspots' was about 1:3 among the DMD patients but too few patients from multigeneration families were included in this study to compare this ratio in isolated versus familial cases. The 75 mutations detected included 15 deletions of a single exon (20%), 57 of more than one exon (76%), and three duplications of several exons (4%). Junctional fragments were found with 8% (6/75) of the deletions.

In the male patient groups the percentage with detectable mutations varied from 50% to 90%, but in the small group of female patients the only mutation detected (by dosage analysis of Southern blots) was in patient F94, the daughter of a Becker patient deleted for exons 45 to 48. No gross chromosomal rearrangements were found in any of the female patients. X chromosome inactivation studies were undertaken but the polymorphisms used in the differential methylation tests were uninformative for S96 and S97. Skewed X inactivation was found in twin S95¹⁰ and patients S98 and S100, while random inactivation patterns within normal

ranges were found in the young BMD carrier F94 using two independent methods.¹³

IMMUNOCYTOCHEMISTRY

The immunocytochemical (ICC) labelling patterns of dystrophin in individual biopsies were very similar with each of the monoclonal antibodies (MAbs) used. Scores for labelling with the rod and C-terminal MAb are given in the appendix. The value of using multiple antibodies is shown in fig 1A–C where a group 4 patient (S60) with a large deletion of exons 13 to 47 shows labelling only with the amino domain and C-terminal antibodies. The amino acids which form the antibody binding site of the rod MAb, Dy4/6D3, are missing. Unequivocal dystrophin labelling (ICC categories 3–5) was found in 24/41 (58%) of the group 1 and 2 DMD biopsies labelled with one or more of the antibodies. In general there was good agreement in the occasional fibres labelled with each MAb but there was not always complete concordance.

Among the male patients, the amount of ICC labelling (Materials and methods, labelling categories 1 to 8) increased according to clinical category so that BMD patients in group 5 showed most labelling (fig 2). Dystrophin labelling among the female patients was very variable but all had some negative fibres, which were found in groups in some cases (S95, S98, and S99). The remaining fibres either showed a continuous spectrum of labelling from near normal to near negative (a pattern found in the younger patients F94, S96, and S97), or there was a high percentage of fibres with normal labelling (60 to 85%) with smaller proportions of fibres with decreased (10 to 20%) dystrophin labelling (patterns found in the older and more mildly affected patients, S98, S99, and S100).

BLOTTING

As with the labelling on sections, the abundance of dystrophin labelling on blots gradually increased as the clinical condition improved from group 1 to group 5 (fig 3). Analysis of variance for all five groups indicates that there is a significant difference ($p < 0.001$) and signi-

Table 1 Exons deleted or duplicated (dup) in different clinical groups (98 independent cases).

Too young (n=7)	1 (n=21)	2 (n=20)	3 (n=9)	4 (n=4)	5 (n=31)	Females (n=6)
None (1)	None (2)	None (4)	None (3)	None (2)	None (5)	None (6)
42–43 dup	8–22	*3–25	*3	*13–47	*45–47 (13)	+ daughter
44	J13–17	J6–9	3–7 (2)	14–26 dup	*45–48 (7)	of a 45–48
45	20–29	8–16 dup	18–29	+ group 4	*45–49 (4)	group 5
*45–49	22–41	*10–13	*42–44	nephew	J45–53	patient
49–50 (2)	36–43	44 (4)	44	with the	*48–59	
	44	45		same dup		
	45 (2)	45–52				
	45–52	46–51				
	45–54	46–55				
	46–52	48–50				
	J46–52	51–55				
	48–50 (2)	51–60				
	J50–51	*52–55				
	51					
	52 (2)					
	J64–66					
86%	90%	80%	Percentage with detectable mutations 67%	50%	84%	0%

Numbers in parentheses indicate the number of subjects with the same mutation.

Deletions marked with * are known to maintain the reading frame.

J = junctional fragment (deletion breakpoint close to, or in, an adjacent exon).

Figure 1 Serial sections from a patient (S60) with a deletion of exons 13 to 47. Dystrophin labelling can be seen with the amino MAb Dy10/12B2 (A) and the C-terminal MAb Dy8/6C5 (C), but the deletion has removed the antibody binding site for the rod MAb Dy4/6D3 (B). (Indirect peroxidase.)

ficant differences were also found when any two individual groups were compared ($p < 0.05$ in all comparisons except that for groups 1 + 2 which was not significant). The appendix summarises the estimates of dystrophin abundance and size for all patients, and blots illustrating the dystrophin labelling in individual patients can be seen in part 2 of this investigation. In every case, we have only considered the largest size polypeptide detected in each patient sample lane.

One important difference between dystrophin labelling on blots and tissue sections was observed: while labelling on sections with the rod and C-terminal antibodies was virtually identical, labelling on blots was always less intense when the C-terminal MAb was used, which meant that labelling with the rod MAb had to be above a certain threshold before C-terminal labelling was detectable (fig 4). All estimates of dystrophin abundance were lower with the C-terminal MAb. Table 2 indicates this difference in 72 male patients (insufficient muscle was available from some patients to produce blots for labelling with more than one

MAb). A significant difference ($p < 0.001$) was found between the estimates of abundance using rod and C-terminal MABs within individual patients.

HISTOPATHOLOGY

Fig 5A shows that the variation in fibre size increased from group 1 to group 5 and analysis of variance was significant ($p < 0.001$) for the five clinical groups. Nevertheless, the differences between any two individual groups were not significant, indicating that there was considerable overlap between the groups. Non-muscle tissue in biopsies is generally fibrous connective tissue (FCT) and fat. The percentage of FCT decreased across groups 1 to 5 (fig 5B) as clinical severity decreased and analysis of variance was highly significant ($p < 0.001$). Significant differences between in-

Figure 2 Immunocytochemical labelling with rod MAb Dy4/6D3. Each biopsy was scored for dystrophin labelling according to the categories described in Materials and methods (1 = negative, 8 = indistinguishable from normal).

Figure 3 Dystrophin abundance (mean, standard error) in different clinical groups estimated from densitometric analysis of blots labelled with the rod MAb Dy4/6D3. Note gradual increase with improved clinical condition. Raw data are in the appendix; group 1 $n = 19$, group 2 $n = 19$, group 3 $n = 8$, group 4 $n = 4$, group 5 $n = 31$. Patients with deletions which remove the antibody binding site, and DMD patient S39 (with atypical BMD-like dystrophin labelling), are not included.

Figure 4 Dystrophin abundance estimated from blots labelled with the rod MAb Dy4/6D3 and the C-terminal MAb Dy8/6C5. A line is drawn at 45° to show where points would lie if the two parameters were identical. The points fall below the line which indicates that all estimates of dystrophin abundance were lower with the C-terminal MAb than with the rod MAb.

dividual groups were found only between groups 1 and 4 ($p < 0.05$) or 1 and 5 ($p < 0.05$). In contrast to FCT, the percentage of fat showed extensive variation without regard to clinical group (fig 5C). Features which indicated acute histopathological changes (hyaline, necrotic, or regenerating fibres) were very variable, but showed a downward trend as clinical severity lessened from groups 1 to 5 (fig 5D). Conversely, although some biopsies from each group showed features of chronic change (split or ring fibres), the highest scores were found in group 5 patients (fig 5E). Grouped (rather than isolated) fibres showing degeneration and regeneration were found in group 5 patients who presented to the clinic before 10 years of age. No obvious relationship was found between any histopathological feature and dystrophin expression that did not also correlate with clinical severity or the age at which the biopsies were taken (range 7 weeks to 64 years).

CREATINE KINASE

An apparent relationship was found between the level of serum creatine kinase (CK) activity at initial presentation to the hospital and the location of certain deleted exons. Relatively low CK values coincided with the cluster of deletions (45–47, 45–48, 45–49) found in

group 5 Becker patients. However, high CK values were found in patients from all clinical categories (including group 5) who presented to the clinic before 7 years. Thus, as expected, the true relationship was between CK level and age at presentation rather than with the missing exons or ultimate clinical severity.

Discussion

SELECTION OF PATIENTS

The patients in this study were selected for inclusion only if they had been examined in Newcastle, had undergone a muscle biopsy, and had provided a blood sample for DNA analysis. They represented the first 100 such patients that we were able to collect, and in fact constituted a reasonably typical sample of the clinical spectrum of patients with Xp21 muscular dystrophy seen in the Northern Region of England. The number of DMD and BMD patients was about equal and this was in agreement with new figures for disease prevalence (the number alive on a particular day) for the region.²⁷ The number of female patients included was small and this reflects the number of such cases seen at our hospital. However, our cohort included a disproportionately large number of sporadic cases. In general, one third of cases of DMD (though not of BMD where the genetic fitness is not zero) would be expected to come from multigeneration families, one third from new mutations arising in the mother (producing brother pairs plus some isolated cases), and one third from new mutations arising in isolated males.²⁸ In recent years, however, improved genetic counselling has reduced the number of affected boys born into families with a history of Duchenne muscular dystrophy. In our cohort 14% of males came from multigeneration families, 13% from brother pairs, and 73% were sporadic cases. This reflects the fact that biopsies were taken from all isolated cases whereas only the first presenting member in a family was usually subjected to this procedure. Many cases were excluded because we had biopsy material stored in the archive but no blood sample, patients having died or moved out of the area. Conversely, blood samples were available from some families but no corresponding biopsy material was available. In almost all cases where more than one family member was affected, there was complete agreement between the different family members for clinical and genetic analyses. The only exception to this was a group 3 intermediate D/BMD patient, B50, who had a more severely affected younger brother (with the same deletion of single exon 44) who was wheelchair bound at 8 years and died aged 18 years. Although a few pairs of brothers with discordant clinical phenotypes have been seen in the clinic over the last 30 years, lack of a muscle sample from both brothers prevented inclusion of such cases in the investigation.

The patient cohort spans a very wide range of disease severity: onset of symptoms from birth to 33 years with loss of independent mobility from 6.3 years to 59 years, if at all. The age at biopsy, which has some bearing on all the protein analyses and histopathological data,

Table 2 Dystrophin abundance estimated from densitometric analysis of blots labelled with antibodies recognising epitopes in the mid rod and C-terminal domains.

Clinical group	Rod domain		C-terminus	
	MAb Dy4/6D3		MAb Dy8/6C5	
	Range	Mean (SD)	Range	Mean (SD)
1 n = 17	0–26	6 (9)	0–8	1 (2)
2 n = 16*	0–30	14 (11)	0–7	2 (3)
3 n = 7	15–40	28 (8)	0–9	3 (4)
4 n = 4	33–90	51 (27)	8–53	23 (21)
5 n = 28	50–96	71 (13)	13–80	31 (15)

Aliquots from the same muscle homogenates were used for both blots.

* Patient S39 excluded as having atypical BMD-like dystrophin abundance.

Figure 5 Histological analysis of biopsies in different clinical groups. Normal upper limits (nul) are indicated by dashed lines. A=fibre size variation (nul=3-fold), B=percentage of fibrous connective tissue (nul=12%), C=percentage of fat (nul=0.5%), D=acute changes index (cumulative score for hyaline fibres, necrotic fibres, regenerated fibres), E=chronic changes index (cumulative score for ring fibres and fibre splitting). See Materials and methods for details.

ranged from 7 weeks to 64 years. The current age range was 4 years to 66 years with many patients being attended by the same clinician throughout their lives. All of these age ranges exceed those in previous large studies,^{34,29} as does the number of different analyses undertaken.

DISTRIBUTION OF DELETIONS

The percentage of deletions/duplications detected among the male patients (81.5% overall) is much higher than the 55 to 65% which has been reported in other investigations.^{5,6,30-32} However, in studies where the clinical diagnosis has been confirmed by dystrophin analysis the percentage of patients with detectable mutations is higher. Thus Beggs *et al*²⁹ and Specht *et al*³³ found that over 80% of patients classified as BMD or DMD had detectable mutations, in agreement with our data. Dystrophin analysis was not performed on all patients in the first major gene mutation surveys and it is possible that some patients who were included in these studies did not really have DMD or BMD, which would result in the apparent deletion rate being lower than it actually was. It has been estimated that up to 12% of patients diagnosed as having DMD, but lacking an X linked family history, may have forms of autosomal recessive MD characterised by normal dystrophin labelling on sections,³⁴ and other reports indicate that patients may be misdiagnosed as DMD without dystrophin analysis.³⁵⁻³⁹

In this study we found no difference in the proportion of sporadic versus familial cases who had mutations (deletions or duplications), in contrast with the results of Passos-Bueno *et al*.⁴⁰ This is almost certainly because of the

relatively large proportion of isolated cases in this study, a suggestion supported by our finding that the ratio of proximal:distal deletions was 1:3 among our DMD patients. In a large two centre study of 473 DMD patients, Passos-Bueno *et al*⁴¹ found that the proximal:distal ratio was 1:3 in isolated cases and 1:1 in familial cases.

DYSTROPHIN ABUNDANCE

In this study we found a significant difference between the apparent abundance of dystrophin on blots labelled with the rod MAb, Dy4/6D3, and with the C-terminal MAb, Dy8/6C5. However, labelling on sections was very similar with the rod and C-terminal MAb. It seems likely that the epitope recognised by Dy8/6C5 is very easily lost in the strenuous processing (homogenisation, boiling, centrifugation) required for Western blotting. In contrast, the preparation of unfixed frozen sections is a more gentle procedure and labelling with rod and C-terminal MABs on sections was virtually identical, suggesting that the C-terminal MAB is not only reacting with a subpopulation of dystrophin molecules, such as might be generated by alternative splicing.⁴² The C-terminus is prone to degradation^{18,25} and the binding of a monoclonal antibody is even more vulnerable to slight changes in polypeptide conformation than a polyclonal antiserum.

All the biopsy samples were labelled with the rod MAB, Dy4/6D3, and the relationship between labelling on sections and labelling on blots was very close for this antibody. We therefore used blots labelled with this MAB for comparison with genotype and clinical phenotype. The precise techniques and reagents used to estimate the abundance of dystrophin from blots vary from laboratory to laboratory, and therefore absolute figures for abundance are not easily compared between centres. Nevertheless, the range of values for DMD and BMD within any centre might be considered. We detect dystrophin in a higher proportion of DMD patients than other laboratories^{3,29,43} and at a higher abundance. Although there was variation between subjects within each group (appendix), the abundance of dystrophin increased significantly from group 1 to 5 and this difference was gradual. All of our DMD patients in groups 1 and 2 had lower dystrophin levels than those found in any BMD patient from group 5. In contrast, the results of Hoffman *et al*³⁴ and Beggs *et al*²⁹ indicate that dystrophin was undetectable in the vast majority of DMD cases, and although isolated DMD cases with values of up to 10% of normal have been reported,²⁹ so have BMD cases with values as low as 5%.⁴ When dystrophin was detected in DMD biopsies the size was within the range found in BMD patients, as we have found.^{15,24} Dystrophin abundance among the majority of BMD cases was above 30%,^{4,29} a value which is similar to our minimum value for BMD patients. The results of Bulman *et al*⁴³ diverge from those of ourselves and Hoffman *et al*^{4,29} in that only truncated dystrophin molecules were detected in DMD patients, with abundances of

up to 17% of normal. Appropriately sized dystrophin was detected in two BMD patients with an abundance of 14 to 21% of normal, but results from a larger number of BMD cases would need to be reported before comparisons could usefully be made.

In this first part of our report we have described our methods, reviewed the patient cohort, and summarised the trends we observed across the clinical spectrum. In part 2 we will consider genotype and phenotype in more detail and report the correlations found within individual patients. In part 3 we use the data obtained from this study of patients with a wide range of disease severity to review which factors or types of analytical techniques appeared most useful for differential diagnosis and prognosis.

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Appendix Summary of data on the 100 patients in this study.

Patient	Clinical group	Age at				Deleted exons	Reading frame	Predicted dystrophin sizes [A]/[B]*	Observed dystrophin size	Dystrophin abundance (Rod/C-term)	ICC (Rod/C-term)	IQ V, P, Full scores
		1st symptoms	Biopsy	Lost mobility	Current							
S1	TY	1-5	1-7	—	5	44	2/3 out	245/421	418	18/0	3/3	—
S2	TY	2-5	2-9	—	4	42-43 dup	3/2 out	(229)/440	418	5/0	3/-	—
S3	TY	2-5	3	—	5	45-49	3/3 in	398	401	72/35	5/5	—
S4	TY	2-5	3-6	—	5	49-50	3/1 out	275/419	—	0/0	1/-	—
S5	TY	3-5	3-8	—	5	None	—	427	427	3/0	3/3	—
S6	TY	2-5	4	—	5	49-50	3/1 out	275/419	427	7/0	4/4	—
S7	TY	3	5-5	—	6	45	3/2 out	251/420	401	21/3	4/4	—
F8	1	Birth	0-1	7-2	Died 14	48-50	3/1 out	268/414	410	5/0	1/-	75, 100, 89
F9	1	2	4	7-3	8	52	3/2 out	294/422	—	0/-	2/-	57, 68, 59
F10	1	1	5	9	Died 18	44	2/3 out	245/421	418	6/0	3/3	75, 82, 76
B11	1	2	3-1	8-2	11	20-29	1/3 out	92/362	—	0/0	1/-	—
B12	1	2	4-7	8-1	10	None	—	427	427	8/-	4/4	<45, <45, <40
B13	1	5	6	8-8	11	51	1/3 out	286/418	418	20/5	4/4	—
S14	1	0-5	1-8	8-9	11	46-(52)J	2/?	255/(387)	—	0/0	1/-	64, 72, 66
S15	1	0-5	1-8	7-8	15	(64-66)J	?	?(360)	—	0/0	1/1	66, 86, 80
S16	1	2-5	3-3	—	6	36-43	3/2 out	(194)/378	418	10/0	3/-	—
S17	1	2-5	3-8	6-7	7	45-52	3/1 out	251/380	—	0/0	1/1	108, 132, 121
S18	1	3	3-8	8	9	50-51J	3/?	278/(410)	410	22/-	2/-	105, 90, 97
S19	1	0-5	3-8	6-3	7	48-50	3/1 out	268/414	—	0/0	3/3	—
S20	1	3	4-4	8-2	14	52	3/1 out	294/422	—	0/0	4/-	64, 68, 67
S21	1	3	4-5	—	7	45	3/2 out	251/420	406	26/0	3/3	102, 81, 91
S22	1	4-5	5-5	8-9	20	46-52	2/1 out	256/387	—	0/0	2/-	—
S23	1	3	5-6	8-7	17	45	3/2 out	251/420	410	26/8	3/3	92, 107, 100
S24	1	3	6	8-8	Died 16	45-54	3/2 out	249/366	—	0/0	1/-	76, 67, 69
S25	1	4	6-5	9	Died 12	22-41	1/3 out	109/314	—	0/-	1/3	80, 88, 84
S26	1	3	7-2	9	10	(13-17)J	?	57/(401)	—	0/0	2/-	84, 82, 81
S27	1	5	7-3	9	11	8-22	1/3 out	28/360	—	0/0	1/-	—
S28	1	4-5	7-8	8-9	18	None	—	427	427	4/0	3/3	84, 84, 82
F29	2	2	2-5	11-5	Died 16	None	—	427	427	25/7	4/-	—
F30	2	3-5	4-2	9-7	10	None	—	427	371	15/0	2/-	59, 52, 52
F31	2	1-5	8-2	11	12	44	2/3 out	245/421	406	17/0	4/3	72, 91, 80
B32	2	2	7	—	Died 11	45	3/2 out	251/420	414	3/0	4/4	82, 70, 74
B33	2	3	8-3	11-9	12	51-55	1/3 out	283/392	384	25/3	4/3	98, 106, 102
S34	2	1	1-9	10-6	11	46-51	2/3 out	256/391	—	0/0	1/1	69, 87, 76
S35	2	3	3	9-3	17	48-50	3/1 out	268/414	410	4/0	2/2	—
S36	2	2-5	3-5	—	8	46-55	2/3 out	258/365	—	0/0	2/3	103, 100, 101
S37	2	2-5	3-6	9-6	10	51-60	1/3 out	284/379	363	22/0	4/-	74, 84, 80
S38	2	2-5	3-9	11-7	Died 17	8-16 dup	1/3 out	80/433	418	10/0	3/3	81, 115, 110
S39	2	3	4-5	9-3	Died 18	3-25	3/3 in	298	299	58/30	5/5	118, 116, 119
S40	2	2-5	5	9-5	20	None	—	427	—	0/-	1/-	(University)
S41	2	4	5-1	10-4	16	44	2/3 out	245/421	410	16/0	3/3	68, 81, 72
S42	2	2-5	5-2	10-8	11	45-52	3/1 out	251/380	—	0/-	2/-	75, 71, 71
S43	2	3	6-7	10-4	14	52-55	3/3 in	401	—	0/0	3/2	45, 60, 55
S44	2	5	6-9	11-5	12	6-9J	3/?	14/(404)	384	16/-	2/2	90, 90, 89
S45	2	5	7-3	—	9	None	—	427	418	7/0	3/-	111, 100, 105
S46	2	3	7-4	10-8	Died 24	10-13	3/3 in	402	385	28/7	4/4	94, 104, 98
S47	2	6-5	7-5	10	17	44	2/3 out	245/421	401	30/6	5/5	73, 90, 84
S48	2	6	8	11-3	18	44	2/3 out	245/421	393	20/0	3/3	45, 78, 64
B49	3	2	10	12-6	19	42-44	3/3 in	408	392	25/0	5/-	114, 96, 106
B50	3	10	10	13-9	25	44	2/3 out	245/421	410	25/0	3/-	105, 104, 105
S51	3	3-5	3-6	—	11	3-7	3/1 out	5/406	401	31/8	4/4	79, 98, 87
S52	3	3	4	12-7	16	18-29	2/3 out	84/353	—	0/0	1/2	—
S53	3	5	8-2	12-3	21	None	—	427	—	27/3	4/4	70, 71, 70
S54	3	4	8-5	14	14	3-7	3/1 out	5/406	401	28/-	5/-	95, 88, 91
S55	3	7	10	14-7	17	3	3/3 in	423	418	33/9	5/5	94, 118, 105
S56	3	9	10-5	12-1	17	None	—	427	427	40/3	3/3	98, 99, 99
S57	3	3	12-5	12-9	13	None	—	427	427	15/0	4/4	78, 72, 73
F58	4	3-5	4	—	16	14-26 dup	3/3 in	504	491	35/10	5/5	—
F59	4	14	32	40	Died 46	14-26 dup	3/3 in	504	491	45/21	5/5	—
S60	4	7	12	17	23	13-47	3/3 in	217	200	0/40	1/8	—
S61	4	4-5	12	16	29	None	—	427	427	90/53	7/7	—
S62	4	4	17	—	25	None	—	427	427	33/8	6/5	75, 82, 77
F63	5	6	10	—	16	45-47	3/3 in	409	401	76/28	6/6	—
F64	5	10	14	—	17	45-48	3/3 in	402	389	70/20	6/6	—
F65	5	10	18	—	26	45-47	3/3 in	409	401	61/30	6/6	—
F66	5	20	31	—	45	None	—	427	427	90/80	8/8	—
F67	5	7	39	—	53	45-48	3/3 in	402	398	74/30	6/-	—
F68	5	23	48	59	62	45-47	3/3 in	409	406	74/40	6/6	(Semi-illiterate)
B69	5	6	13	—	21	45-49	3/3 in	398	397	77/15	6/6	88, 109, 98
B70	5	13	17	—	18	45-47	3/3 in	409	401	55/26	6/6	—
B71	5	13	24	—	29	45-47	3/3 in	409	401	89/48	6/6	—
B72	5	1-5	24	—	24	45-48	3/3 in	402	397	80/18	6/6	—
B73	5	6	39	—	53	45-48	3/3 in	409	392	77/30	7/-	—
S74	5	1	5	—	8	45-47	3/3 in	398	384	53/13	5/5	87, 87, 86
S75	5	5	7	—	15	45-49	3/3 in	409	401	84/23	6/6	101, 106, 103
S76	5	3	7	—	15	45-47	3/3 in	409	401	80/28	5/5	—
S77	5	2	10	—	14	None	—	427	427	96/38	7/7	—
S78	5	9	13	—	20	45-48	3/3 in	402	401	61/22	6/6	80, 92, 85
S79	5	6	14	—	15	45-49	3/3 in	398	393	61/22	6/6	88, 92, 89
S80	5	1-5	15	—	16	45-47	3/3 in	409	401	55/25	6/6	—
S81	5	3	15	—	32	45-(53)J	3/?	(372)	367	84/43	6/6	—
S82	5	7	20	—	34	45-48	3/3 in	402	395	68/27	6/6	—
S83	5	10	20	—	24	45-47	3/3 in	409	401	73/-	6/-	—
S84	5	5	21	—	27	45-49	3/3 in	398	389	57/26	6/6	(ESN)
S85	5	17	22	—	36	45-47	3/3 in	409	401	70/30	6/6	—
S86	5	21	24	—	27	48-59	3/3 in	349	350	97/-	6/-	—
S87	5	28	30	—	35	None	—	427	427	50/20	6/6	—
S88	5	5	35	—	45	45-48	3/3 in	402	401	59/30	6/-	—
S89	5	28	35	—	47	45-47	3/3 in	409	401	85/-	6/6	—
S90	5	5	36	—	42	None	—	427	427	95/73	6/6	—
S91	5	17	42	—	50	None	—	427	427	68/20	7/7	—
S92	5	30	44	—	59	45-47	3/3 in	409	401	65/35	7/7	—
S93	5	33	64	—	66	45-47	3/3 in	409	401	61/22	6/6	—
F94	F	2	3	—	5	45-48	3/3 in	427 + 402	427 + 398	50/28	6/6	—
S95	F	3	8	11	Died 16	None	—	427	—	37/-	6/6	88, 102, 94
S96	F	7	8	—	9	None	—	427	427	77/50	6/6	82, 80, 80
S97	F	6	9	13	15	None	—	427	427	76/-	6/6	102, 105, 103
S98	F	4	24	—	25	None	—	427	427	45/32	6/6	—
S99	F	21	26	—	38	None	—	427	427	80/70	6/6	—
S100	F	17	35	—	45	None	—	427	427	60/45	6/6	—

* = Predicted dystrophin sizes for patients with frameshifting deletions are calculated so [A] = size to premature stop codon, and [B] = size if reading frame immediately restored (see part 2). — = data not available. ? or parentheses = data not certain. dup = exons duplicated rather than deleted. J = junctional fragment. ICC = immunocytochemistry. V,P, = verbal IQ, performance IQ. ESN = classified as educationally subnormal. Patients F58 and F59, and patients F67 and F94 are related.