Collagen VI mutations in Bethlem myopathy
Jöbsis, G.J.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 2.1

Genetic localization of Bethlem myopathy

G.J. Jöbsis, P.A. Bolhuis, J.M. Boers, F. Baas, R.A. Wolterman, G.W. Hensels, M. de Visser

Department of Neurology, Academic Medical Center, Amsterdam, The Netherlands

*Neurology* 1996;46:779-782
Chapter 2.1

Abstract

Bethlem myopathy is a rare autosomal dominant myopathy characterized by slowly progressive limb-girdle muscular atrophy and weakness, and contractures of multiple joints. To identify the genetic localization we used highly polymorphic microsatellite markers in a genome-wide search in six Dutch families. After excluding genetic linkage with 52 markers distributed evenly over the autosomes, significant linkage was present with the 21q22.3 locus PFKL (two-point lod score of $Z_{\text{max}} = 6.86$ at $\theta = 0.03$). There was no indication of genetic heterogeneity. The pattern of recombinations observed with adjacent markers indicated a localization distal to PFKL. Recombination of a marker within the collagen α1(VI) gene (COL6A1) excluded this apparent candidate gene in one of the Bethlem myopathy families. The disease gene is most likely located in the region between COL6A1 and the telomere of chromosome 21q.

Introduction

Bethlem myopathy (early-onset benign autosomal dominant limb-girdle myopathy with contractures) is a hereditary myopathy with slowly progressive muscular atrophy and weakness, and contractures of multiple joints. Onset of weakness is usually either in the neonatal period, presenting with hypotonia, or in early childhood. There is generalized, slight atrophy of the musculature and diffuse, mild weakness, proximal more severe than distal. Involvement of the facial muscles is absent. Nearly all patients have flexion contractures of the interphalangeal joints of the four last fingers, elbows and ankles. Contractures of the metacarpophalangeal joints, wrists, knees, hips, and shoulders are less common. In contrast with X-linked Emery-Dreifuss muscular dystrophy, contractures of the neck and spine are rarely present. The contractures, present from onset of weakness onwards, give little or no functional impairment in most patients. In spite of slowly ongoing deterioration of muscle power, ability to work is preserved until old age. Some patients remain ambulant, often with help of a walking cane, whereas others become wheelchair-dependent for outdoor transportation. There is no cardiac involvement and life expectancy is unaffected. Ancillary investigations such as electrophysiological studies and muscle biopsy indicate a myopathic origin of the disease.

Bethlem myopathy has an autosomal dominant mode of inheritance with complete penetrance. A previous linkage effort failed to detect genetic linkage. In the current study we carried out a genome-wide search with highly polymorphic microsatellite markers in six Dutch families.
Methods

Subjects. Sixty-eight family members (of whom 34 patients) from six pedigrees had blood drawn. Data from Families A to E have previously been published.\textsuperscript{1,3,6} Genealogical investigation could not establish links between the first three pedigrees from the beginning of the eighteenth century onwards. No proper genealogical studies have been undertaken for Families D to F. Pedigree D is of Polish descent and the other families are not known to be related to each other. Except for two new patients, all patients had previously been seen at our department. Variability in severity of weakness as well as nature and site of contractures is present within and between families. Individuals were considered affected on the basis of the presence of weakness and/or contractures. The clinical characteristics are summarized for each family in Table 1.

<table>
<thead>
<tr>
<th>Family</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of patients</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

Weakness and wasting
- upper limb, proximal
  | 5 | 8 | 5 | 4 | 8 | 2 |
- upper limb, distal
  | 5 | 7 | 2 | 4 | 6 | 1 |
- lower limb, proximal
  | 5 | 8 | 5 | 4 | 7 | 2 |
- lower limb, distal
  | 5 | 3 | 3 | 3 | 8 | - |
- neck flexors
  | 5 | 7 | 3 | 3 | 7 | - |

Contractures
| 6 | 8 | 5 | 4 | 7 | 2 |

Reflexes
- reduced or absent
  | 3 | 5 | 3 | 3 | 5 | 1 |

Table 1. Clinical features in 34 patients with Bethlem myopathy

Microsatellite typing. Genomic DNA was extracted from leukocytes. Individuals were genotyped using highly polymorphic microsatellites derived from published sources (GenomeDataBase). Primers for polymerase chain reaction (PCR) were obtained from the Dutch Microsatellite Primerbank (NWO, The Netherlands) or were synthesized on a Cyclone Plus DNA synthesizer (Millipore). PCR, electrophoresis and detection of alleles.
Additional chromosome 21q22.3 markers. The COL6A1 polymorphisms (TaqI and BamHI RFLPs, TaqI and BamHI VNTRs) were detected with cDNA probe P18 (ATCC 61312) as previously described.\textsuperscript{7, 8, 9} COL6A1 VNTRs could not be reliably scored. Because not all meioses were informative for the COL6A1 RFLPs, three intron sequences (intron 3, 10B and 17) of the triple helical domain of COL6A1 were investigated for polymorphisms by means of single strand conformation (SSC) polymorphism analysis.\textsuperscript{10} Primers for intron PCR were selected from the COL6A1 genomic sequence (Genbank S75385S01 through S75385S18) using the PRIMER computer software program. Intron 3 was found to contain a SSC polymorphism (confirmed by sequence analysis) that was used in the construction of a COL6A1 haplotype. A CD18 3' untranslated region polymorphism was detected using SSC as published with minor modifications.\textsuperscript{10}

Linkage analysis. Allelotypes were placed in the pedigrees and analyzed for linkage. Two-point lod scores were obtained by means of the MLINK subprogram of the LINKAGE software package (FASTLINK version 2.2).\textsuperscript{11} Linkage calculations and homogeneity tests (HOMOG program version 3.33)\textsuperscript{12} were performed using the computing facilities of the UK Human Genome Mapping Project Resource Centre. The disease was considered to be autosomal dominant with complete penetrance and a gene frequency of 0.000001. The recombination fractions were taken to be equal in males and females. For COL6A1, a haplotype was constructed from the alleles of four COL6A1 polymorphisms.

Results

Initially, we analysed apparent candidate regions like the dystrophin-like protein (utrophin) gene on chromosome 6q24, the limb girdle muscular dystrophy 1A locus (LGMD1A) on 5q22.3-31.3, LGMD2A on chromosome 15q15.1-q21.1, LGMD2B on chromosome 2p16-p13, and LGMD2C on chromosome 13q12 without detecting linkage. A total of 52 microsatellite markers from all autosomes were examined without finding linkage, thereby excluding roughly 30% of the autosomal genome (two-point lod scores \( Z \approx -2.0 \), data not shown).

Significant linkage was found with the 21q22.3 marker D21S171 (Table 2). Additional markers (ordered from telomere to centromere) COL6A1, CD18, PFKL, D21S1259, D21S49, D21S212, D21S1260, D21S266, HGM14 and D21S211, all showed linkage (Table 2). PFKL yielded the highest pairwise lod score, \( Z_{\text{max}} = 6.86 \) at \( \theta = 0.03 \). The maximum two-point lod score obtained within one family (Family 2) with this marker was 3.76 at \( \theta = 0.00 \). Each marker displayed at least one recombination.
Table 2. Two-point lod scores for six Bethlem myopathy pedigrees and markers (from telomeric to centromeric) on chromosome 21q22.3

<table>
<thead>
<tr>
<th>Marker</th>
<th>Recombination fraction</th>
<th>Z_{max}</th>
<th>( \Theta_{max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>COL6A1</td>
<td>-\infty</td>
<td>6.21</td>
<td>6.27</td>
</tr>
<tr>
<td>Cd18</td>
<td>-\infty</td>
<td>3.31</td>
<td>3.76</td>
</tr>
<tr>
<td>PFKL</td>
<td>-\infty</td>
<td>6.69</td>
<td>6.76</td>
</tr>
<tr>
<td>D21s171</td>
<td>-\infty</td>
<td>2.37</td>
<td>3.93</td>
</tr>
<tr>
<td>D21S1259</td>
<td>-\infty</td>
<td>5.62</td>
<td>5.81</td>
</tr>
<tr>
<td>D21S49</td>
<td>-\infty</td>
<td>3.89</td>
<td>4.66</td>
</tr>
<tr>
<td>D21S212</td>
<td>-\infty</td>
<td>4.15</td>
<td>5.49</td>
</tr>
<tr>
<td>D21S1260</td>
<td>-\infty</td>
<td>5.12</td>
<td>5.8</td>
</tr>
<tr>
<td>D21S266</td>
<td>-\infty</td>
<td>2.82</td>
<td>4.37</td>
</tr>
<tr>
<td>HGM14</td>
<td>-\infty</td>
<td>-0.85</td>
<td>0.95</td>
</tr>
<tr>
<td>D21S211</td>
<td>-\infty</td>
<td>-0.97</td>
<td>0.51</td>
</tr>
</tbody>
</table>

The families were analysed for genetic heterogeneity by means of the HOMOG program. To obtain maximal informativeness of each analysis, the following tightly linked markers were constructed into haplotypes: D21s171 and PFKL (genetic distance ± 2cM), D21S1259 and D21S49 (± 1 cM) and D21S1260 and D21S266 (0 cM).\textsuperscript{13-15} In each analysis the overall maximum likelihood of either homogeneity or heterogeneity was obtained with alpha = 1.00 (alpha is proportion of families showing linkage to the marker). For instance, for COL6A1, the maximum likelihood for alpha = 0.80 was 9.69 x 10^{4} at \( \theta = 0.00 \), whereas the overall maximum likelihood was 1.86 x 10^{5} (\( \theta = 0.05 \) and alpha = 1.00). For COL6A1, D21s171 + PFKL, D21S1259 + D21S49, and D21S212 the overall maximum likelihoods were found at \( \theta = 0.05 \), and for D21S1260 + D21S266 at \( \theta = 0.10 \). These data suggest genetic homogeneity; however, the families are not sufficiently large to prove that only one gene is involved.

The alleles of all tested 21q22.3 markers were constructed into a haplotype to
obtain the most probable localization of each crossover event. Telomeric markers (COL6A1, Cd18 and PFKL) showed fewer recombinations than the more centromeric markers (see Table 2). From this haplotype contraction it was apparent that in one patient (in Family A) all markers including the most distal marker (COL6A1), recombined with the disease. Clinical reassessment affirmed that this individual was indeed affected; he had flexion contractures of the interphalangeal joints. A second blood sample was obtained and DNA extracted from this sample was retested for the COL6A1 polymorphisms, yielding identical results to the first sample.

Discussion

The linkage data of the present study indicate that the Bethlem myopathy locus is located on the distal part of chromosome 21q. A two-point lod score exceeding 3.00 in one family is compelling evidence for linkage, as is the case for Family B with COL6A1, PFKL and D21S212. Because the statistical power of a homogeneity test with linkage data like ours is limited, we cannot conclude definitely that all pedigrees are linked to chromosome 21q22.3. However, the HOMOG results indicate that genetic homogeneity is more likely than heterogeneity.

From the haplotype constructions all families contribute to linkage. Distal markers give rise to fewer recombinations than proximal markers. Taken together with the recombination between the markers and the disease in one patient from Family A, the most likely localization is distal to COL6A1. Located on a telomere, markers are only available at one side of the locus, thus making it difficult to confine precisely by genetic analysis the candidate region. COL6A1 is located on the most distal NotI fragment of chromosome 21q with an estimated size of 0.8 Mb.

The collagen VI genes might be considered good candidate genes for Bethlem myopathy because of the involvement in binding of the basal lamina to extracellular matrix glycoproteins, the expression in skeletal muscle, and the widespread occurrence of contractures. The collagen VI protein consists of three peptides: α1(VI) (COL6A1), α2(VI) (COL6A2) and α3(VI) (COL6A3). The COL6A1 and COL6A2 genes on the distal end of chromosome 21q have probably arisen from a duplication event. On the NotI contiguous map of the IVth International Workshop on Human Chromosome 21 COL6A1 is shown as the most distal gene. This orientation and the recombination with COL6A1 observed in one patient exclude the COL6A1-2 genes as disease genes of Bethlem myopathy. The only identified gene telomeric of the COL6A1-2 cluster on chromosome 21q encodes the β subunit of protein S-100 (S100B). As it is mainly expressed in glial cells and not in skeletal muscle, this gene is not a good candidate for Bethlem myopathy.

In conclusion, the present study has assigned Bethlem myopathy to the telomere of
chromosome 21q. Most likely, the disease gene is an as yet unidentified gene distal of COL6A1. Bethlem myopathy displays typical clinical characteristics with a virtually constant expression. Nonetheless, there are some reports of limb-girdle myopathies with contractures displaying some atypical features.\textsuperscript{22,23} The identification of the genetic defect of Bethlem myopathy will help to delineate the nosological classification of those families.

Acknowledgements

We are indebted to the family members who participated in this study. We like to thank Dr. G.J. te Meerman, Department of Medical Genetics, University of Groningen, The Netherlands, for help with the HOMOG program. Supported in part by grants from the Prinses Beatrix Fonds and the Schuhmacher-Kramer Foundation.

References


Chapter 2.2

Bethlem myopathy: linkage to a VNTR within the COL6A1 gene on chromosome 21q


Department of Neurology, Academic Medical Center, Amsterdam, The Netherlands; National Public Health Institute, Department of Human Molecular Genetics, Helsinki, Finland; Department of Biology, University of Padua, Padua, Italy; Department of Neurology, University of Helsinki, Helsinki, Finland; Istituto Ortopedico Rizzoli, Bologna, Italy
Abstract

Bethlem myopathy is a benign, early-onset myopathy characterized by weakness and contractures of multiple joints, and an autosomal dominant mode of inheritance. We have reported linkage to the telomere of chromosome 21q in six Dutch families. This region contains two apparent candidate genes, encoding the α1 (COL6A1) and α2 (COL6A2) subunits of collagen VI, an anchoring protein in the basal lamina of striated muscle. A French-Canadian kindred does not link to the COL6A1-COL6A2 cluster but to the COL6A3 region on chromosome 2q. By PCR amplification of a VNTR within the COL6A1 gene, we confirm linkage to the COL6A1-COL6A2 cluster in three additional kindreds, two from Italy and one from Finland. Joint two-point lod scores exceed 8. The families do not share a common allele of the COL6A1 VNTR. These findings provide further support for the role of collagen VI in Bethlem myopathy.

Introduction

The category of limb-girdle syndromes contains an entity characterized by moderate weakness, contractures of multiple joints, absence of cardiac involvement, onset in early childhood, a benign course, and an autosomal dominant mode of inheritance. Initially described in three Dutch families by Bethlem and van Wijngaarden, this entity has become known as Bethlem myopathy. Several kindreds with Bethlem myopathy have been reported, all displaying remarkably uniform features.

A genome-wide search in six families from the Netherlands yielded linkage with markers in the telomeric band of chromosome 21q. Two candidate genes have been mapped to the region, COL6A1 and COL6A2, encoding two subunits of collagen VI. However, a French-Canadian kindred, clinically indistinguishable from the Dutch families, did not link to this locus. Instead, this kindred showed linkage to a region on chromosome 2q containing the gene encoding the third subunit of collagen VI, i.e. COL6A3.

In this study we analyse three additional Bethlem myopathy kindreds for linkage to the COL6A1-COL6A2 cluster. Apart from BamHI and TaqI RFLPs, a cDNA probe representing the triple helical (TH) domain of COL6A1 has been reported to detect two informative VNTRs, also after either BamHI or TaqI digestion. To our experience, both VNTRs were difficult to visualize and therefore, we designed a PCR assay to genotype the COL6A1 VNTR.
Confirmation of linkage

Figure 1. Participating sibships of three Bethlem myopathy pedigrees. Individuals genotyped are indicated with a dot below the pedigree symbol. Gray pedigree symbol refers to individual with equivocal clinical findings.

Patients and Methods

The pedigrees are depicted in Figure 1. Clinical descriptions have been published for Families 1 and 2, and Family 3. After obtaining informed consent, genomic DNA of 44 family members was isolated from peripheral blood using standard protocols.

To identify the location of the COL6A1 VNTR, genomic DNA of the COL6A1 TH domain was amplified using the Expand™ Long Template PCR System (Boehringer Mannheim) following the manufacturer's guidelines. The following oligonucleotides were used as primers: COL6A1F1 (5'-CACCGTCTCCTCCTGTGTGTGTTCCAGGGAAGAC-3', starting at position -25 of exon 3 of the TH domain), COL6A1F2 (5'-CTCCCTCCATGTCCTCCACTCAGGTGGC-3', pos. -24 of exon 11), COL6A1R1 (5'-CTGGGAAGACCGGGAGTGCAACAACGCTG-3', pos. -1 of exon 9), COL6A1R2 (5'-GGGACCCCTCATCGCTCGGTAGCTCTTTagg-3', pos. 51 of exon 13), and COL6A1R3 (5'-GCCCCTGCACTGACGAGGAGCAGCCTGGTATA-3', pos. 3 of exon 17); sequences were derived from Genbank (S75385S01-16). After 0.8 % agarose gel electrophoresis, PCR products were
visualized by ethidium bromide staining. Genomic DNA was allelotyped for the \textit{COL6A1} VNTR (primers \textit{COL6A1F1} and \textit{COL6A1R1}); alleles were scored from the agarose gels. Segregation of the VNTR was investigated for the three Bethlem myopathy families at study and 73 persons from seven other kindreds.

Genotypes were analysed using maximum likelihood methods to perform pairwise linkage analysis with the MLINK program of the FASTLINK package\textsuperscript{11} with complete disease penetrance and assumed equal frequencies of six alleles. One individual from Family 2 was scored as unknown (see below).

<table>
<thead>
<tr>
<th>Family</th>
<th>0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.44</td>
<td>1.41</td>
<td>1.29</td>
<td>1.14</td>
<td>0.82</td>
</tr>
<tr>
<td>2</td>
<td>3.01</td>
<td>2.96</td>
<td>2.77</td>
<td>2.51</td>
<td>1.95</td>
</tr>
<tr>
<td>3</td>
<td>3.65</td>
<td>3.58</td>
<td>3.32</td>
<td>2.98</td>
<td>2.25</td>
</tr>
<tr>
<td>Σ</td>
<td>8.1</td>
<td>7.95</td>
<td>7.38</td>
<td>6.63</td>
<td>5.02</td>
</tr>
</tbody>
</table>

\textbf{Table 1.} Two-point lod scores for three Bethlem myopathy kindreds and a \textit{COL6A1} VNTR

Results

Genomic amplification of almost the entire TH domain rendered products of roughly 10 kb displaying some variation in length. \textit{KpnI} digestion of the PCR product pinpointed the length polymorphism between exon 3 and exon 7.\textsuperscript{12} To enhance allele separation, more approximate VNTR flanking primers were used; primer combination \textit{COL6A1F1} and \textit{COL6A1R1} amplified a fragment of about 2.5 kb with length variation (Figure 2). At most two different amplification products were observed for each person, whereas six different alleles were found in total. Alleles segregated in a Mendelian fashion without instability. The observed rate of heterozygosity was 77\% in 117 individuals from 10 families.

Table 1 displays the results of the lod score calculation for linkage between the \textit{COL6A1} VNTR and Bethlem myopathy. For one person from Family 2, the marker recombined with the attributed disease status. Resampling yielded identical VNTR alleles. However, detailed clinical examination at the time of resampling revealed that the previously attributed disease status might not have been correct. In brief, the clinical details were as follows;

This 32 year old man (unaffected individual III-1 from Family 2 in the original
Confirmation of linkage

had no history of weakness. Equivocal findings consisted of minimal weakness of the orbicularis oculi and oris muscles and the left extensor digitorum muscle, and ambiguous flexion contracture of the distal interphalangeal joints. Muscle computer-tomography showed no definite muscle degeneration.

Figure 2. Example of allele segregation of the \textit{COL6A1} VNTR.

Discussion

The investigated polymorphism is located between exon 3 and exon 7 of the TH domain of \textit{COL6A1}. Previously, a \textit{COL6A1} VNTR has been described detected with a cDNA fragment encoding the TH domain (from exon 8 to exon 17) on TaqI Southern blots (GDB G00-155-404). Given the genomic map of the region and a TaqI site in exon 6, the published VNTR is most likely identical to the present polymorphism. The addition of Pwo DNA polymerase with proof-reading activity to the PCR enables reliable amplification of the VNTR previously typed by Southern blotting.

All studied families display linkage to \textit{COL6A1}. The families do not share a common disease allele, suggesting the mutations have occurred independently. The absence of a common allele could, however, be due to meiotic instability of the VNTR. Like previously found in the Dutch kindreds, recombinations were present in the Italian families with more proximally located markers \textit{D21S171}, \textit{PFKL}, and \textit{D21S212} (results not shown).

The uncertain clinical status of one person in Family 2 hampers interpretation of the genetic data of that family. Weakness of the facial musculature is unusual for Bethlem myopathy. It has however been described in an other Italian Bethlem myopathy kindred. Young adult Bethlem myopathy patients display relatively little weakness. Given the
uncertainty of the disease status of this person, he was scored as unknown in the linkage analysis. Multiple recombinations with \textit{COL6A3} polymorphisms ruled out a localisation at chromosome 2q in Family 2 (results not shown).

In the original report on Family 3, Somer et al. did not make a definite diagnosis of Bethlem myopathy.\textsuperscript{10} Features considered atypical of Bethlem myopathy included onset in adolescence for most individuals and hypertrophic calves in one patient. Linkage to the \textit{COL6A1-COL6A2} cluster could be taken as confirmation of this family being afflicted with Bethlem myopathy, alternatively one could argue for it being an allelic but nosologically separate entity.

Collagen VI, present in the extracellular matrix of many tissues including skeletal muscle\textsuperscript{16} forms microfibrils that are believed to play a role in cell-matrix interactions.\textsuperscript{17} These interactions may involve the dystrophin-associated proteins via laminin in the basal lamina of skeletal muscle.\textsuperscript{18} Alterations of these proteins have been reported in several muscular dystrophies, the association of Bethlem myopathy with collagen VI is in line with these findings.

Acknowledgements

We are indebted to the family members who participated in this study. Supported in part by grants from the Prinses Beatrix Fonds, the Schuhmacher-Kramer Foundation and Italian Telethon.

References


Chapter 2.3

Collagen VI mutations in Bethlem myopathy, an autosomal dominant myopathy with contractures

G.J. Jöbsis, H. Keizers, J.P. Vreijling, M. de Visser, M.C. Speer, ¹ R.A. Wolterman, F. Baas, P.A. Bolhuis

Department of Neurology, Academic Medical Center, Amsterdam, The Netherlands;
¹Division of Neurology, Duke University Medical Center, Durham, United States of America

Nature Genetics 1996;14:113-115
Abstract

Among the diverse family of collagens, the widely expressed microfibrillar collagen VI is believed to play a role in bridging cells with the extracellular matrix. Several observations imply substrate properties for cell attachment as well as association with major collagen fibres. Previously, we have established genetic linkage between the genes encoding the three constituent \( \alpha \) chains of collagen VI and Bethlem myopathy. A distinctive feature of this autosomal dominant disorder consists of contractures of multiple joints in addition to generalized muscular weakness and wasting. Nine kindreds show genetic linkage to the \textit{COL6A1-COL6A2} cluster on chromosome 21q22.3 (refs 3, 4, Chapter 2.2) whereas one family links to markers on chromosome 2q37 close to \textit{COL6A3}. Sequence analysis in four families reveals a mutation in \textit{COL6A1} in one and a \textit{COL6A2} mutation in two other kindreds. Both mutations disrupt the Gly-X-Y motif of the triple helical domain by substitution of Gly for either Val or Ser. Analogous to the putative perturbation of the anchoring function of the dystrophin-associated complex in congenital muscular dystrophy with mutations in the \( \alpha_2 \) subunit of laminin, our observations suggest a similar mechanism in Bethlem myopathy.

We analysed \textit{COL6A1} and \textit{COL6A2} cDNA from four Bethlem myopathy families, with linkage to the \textit{COL6A1-2} cluster, by PCR amplification and sequencing. The amplification products showed no differences in length from the expected size. For \textit{COL6A1}, we identified a heterozygous missense mutation c.962G>T (G286V) in the triple helical domain, present in both cDNA and genomic DNA of a patient from Family B. Sequence analysis of both parents and restriction analysis of the remaining 18 family members (loss of a NlaIV site) showed perfect co-segregation of the mutation with the Bethlem myopathy trait (Figure 1a). All patients were heterozygous for the mutation. The base substitution was not present in 50 unrelated controls. The predicted change of amino acid entails a disruption of the triple helix defining Gly-X-Y motif. We did not detect the mutation c.962G>T (G286V) in six additional Bethlem myopathy pedigrees tested.

For \textit{COL6A2}, two Bethlem myopathy kindreds (Families D and E) shared a heterozygous missense mutation c.898G>A (G250S) in the triple helical region (Figure 2). This mutation also resulted in a disruption of the Gly-X-Y motif, co-segregated with the Bethlem myopathy trait as studied by loss of a SphI restriction site (Figure 1b), and was absent in 50 controls. It was not present in six other kindreds. In addition to these mutations, we have identified other nucleotide changes (results not shown). These alterations are thought to be irrelevant to Bethlem myopathy by virtue of being either silent, homozygously present or also present in control samples.
Mutations

Figure 1. Segregation of COL6A1 and COL6A2 mutations visualized by restriction analysis. a) COL6A1 mutation c.962G>T (G286V) resulting in loss of a NlaIV site in a sibship of Family B (lanes 2-7), lane 1 not digested PCR product. b) COL6A2 mutation c.898G>A (G250S) with loss of Syl1 site shown for a part of Family E (lanes 2-7), lane 1 not digested PCR product.

From the genetic linkage data and the subsequent mutation analysis we conclude Bethlem myopathy is a collagen VI disorder. Collagen VI mutations have not been reported before, neither have neuromuscular diseases based on collagen aberrations. The mutations, present in one allele as expected in an autosomal dominant disorder, segregate with the disease. It is unlikely the base substitutions are merely linked polymorphisms because the predicted amino acid changes involve glycine substitutions in the collagenous domain. They do not arise in 100 control chromosomes. Our observations imply genetic heterogeneity as the two mutations are not present in six additional, 21q22.3 linked Bethlem myopathy families.

Of the four kindreds studied, we have identified a COL6A1 or COL6A2 mutation in three. The failure to find a mutation in Family C might be due to a restriction of the method used. The mutation analysis was carried out on PCR amplified cDNA, and aberrant splicing or mutations in regulatory sequences can escape detection. For COL6A1, but not COL6A2, we have found heterozygosity for a polymorphism at pos. 2784, indicating that both alleles of this gene are transcribed at least in part. The alternative possibility that COL6A3 is the disease gene in this family instead of either COL6A1 or COL6A2, is made unlikely by multiple recombinations with D2S345, a tightly COL6A3 linked marker5 (results not shown).
Disruption of the triple helix motif, as we have found in both COL6A1 and COL6A2 for Bethlem myopathy, is commonly encountered in other collagen disorders, like osteogenesis imperfecta (OMIM 120150/120160), Ehlers-Danlos syndrome type IV (OMIM 120180) and in Alport syndrome. The sequelae of such alterations comprise altered proteolytic cleavage, poor post-translational processing, and disturbed formation of both collagen monomers and of larger multimeric assemblies. Collagen VI is not only present in the endomysium and perimysium of skeletal muscle, but also in many other tissues. The features of Bethlem myopathy are however restricted to skeletal muscle. This divergence, which is similar to other collagen disorders like e.g. Alport syndrome, awaits proper explanation.

We suggest the disease mechanism either consists of an effect on the differentiation process of myoblasts or, more likely, interferes with the structural organization of the extracellular matrix. Synthesis of collagen VI is increased considerably upon differentiation of mesodermal cells like myoblasts. Differentiation may depend on the presence of collagen VI and therefore, altered molecules or a decreased amount may interfere with development of myofibres. The features of Bethlem myopathy can be present from birth onwards, manifesting in some patients as congenital torticollis, contractures or neonatal floppiness, suggesting a congenital nature. Many patients however do not develop significant weakness before the first years of life and this tends to worsen gradually, especially in adulthood.

Components of the dystrophin-associated complex are mutated in several muscular dystrophies, like dystrophin in Duchenne and Becker muscular dystrophy, and of various sarcoglycans in limb girdle muscular dystrophies. Linking the cytoskeletal
Mutations

protein actin with the extracellular matrix, the dystrophin axis is secured to the basal lamina by the α subunit of laminin. Mutations in the laminin α2-chain gene (LAMA2) are present in merosin-negative congenital muscular dystrophy\textsuperscript{23} and also in the dy\textsuperscript{2J}/dy\textsuperscript{2J} mouse.\textsuperscript{24} The identification of collagen VI mutations in Bethlem myopathy seems to extend the fixation system one step beyond, from laminin into the surrounding endomysial connective tissue.

Methods

RNA isolation and reverse transcription. RNA was extracted from cultured skin fibroblasts or a deep frozen muscle biopsy specimen of four affected individuals from Bethlem myopathy kindreds linked to 21q22.3 markers;\textsuperscript{4,4} Families B,\textsuperscript{6} C,\textsuperscript{6} D,\textsuperscript{9} and E;\textsuperscript{8} and from a control subject using guanidine isothiocyanate. Total RNA (2 µg) was used for reverse transcription with random primers and reverse transcriptase (Gibco BRL) using standard protocols.

COL6A1 and COL6A2 cDNA sequence analysis. The entire coding sequence of both genes was PCR amplified in multiple, overlapping fragments, ranging from 700 to 1600 bp in length. In case of COL6A1 (joint GenBank sequences HSCOLIN, HUMCOLTHA and HSCOLIC; 4115 bp with ATG at 49 and TAG at 3135) the most 5' forward primer represented nucleotides 4-29 and the most 3' reverse primer nucleotides 3230-3256. For COL6A2 (joint Genbank sequences HUMCOL6A2A from the start of exon 1 at 2141, HUMCOL6A2B, HUMCOL6A2C, HUMCOLTHB and HSCOL2C1; 3394 bp with ATG at 91 and TAG at 3147) the ultimate primers represented nucleotides 36-60 and 3179-3207 for amplification of the major N-terminal (AS7) and C-terminal (α2C2) splice variant. The lesser N-terminal (AS5, AS5a and AS5b) and C-terminal (α2c2a and α2c2a') splice variants were not amplified and thus not analyzed. Both strands of the amplification products were cycle sequenced using internal primers labelled with γ\textsuperscript{32P} and Sequiterm DNA polymerase (Epicentre Technologies) according to standard protocols. Primers for PCR amplification and cycle sequencing are available upon request. Deviations from the published COL6A1-2 sequence (as above plus S75385S01-18, S75425S01-19, R36608, R82996, and T80711) and from the sequence of a control sample was confirmed by cycle sequencing of PCR products of genomic, leukocyte derived DNA from the same patient.

Segregation analysis. Segregation was studied by restriction analysis of PCR amplified genomic DNA of family members. In case of the COL6A1 mutation G286V, the primers 5'-TGGGTACCAGGGAATGAAGG-3' (pos. 27, exon 3 of TH domain) and 5'-CCTCACTCACCTTCTCCCCA-3' (pos. 37, exon 4 TH domain) rendered a 155 bp fragment with one variable (G286V dependent) and two constant sites for NlaIV. For the COL6A2
mutation G250S, primers 5'-GCCTCGATGTACTCTTTCTCTGCT-3' (pos. -29 exon 6) and 5'-GGGTCTCCCTGTGAAGGACA-3' (pos. 8 exon 7) rendered a 170 bp fragment with one variable (G250S dependent) site for Styl. Six additional Bethlem myopathy kindreds -that either link to the COL6A1-6A2 gene cluster, Families A, 1 and 2, 10 and 3, 26 (see Chapters 2.1 and 2.2) or are too small to establish linkage, Families F and 4 (both unpublished),- not included in the cDNA sequence analysis due to lack of fibroblasts of patients, were screened for these mutations by similar restriction analyses. Furthermore, 50 unrelated controls from the same population background were checked for each mutation.

**Linkage analysis COL6A3 locus.** Eight members of Family C were genotyped for D2S345 (GDB G00-246-292) using standard methods. Genotypes were analysed for pairwise linkage with the MLINK program of the FASTLINK package 25 with assumed full penetrance, disease frequency of 0.000001 and equally distributed frequencies of eight alleles.

**Acknowledgements**

We are grateful to the Bethlem myopathy patients who agreed to undergo a skin biopsy for the purpose of this study. This work was partly funded by grants from the Prinses Beatrix Fonds, the Schuhmacher-Kramer Foundation, and the AMC profiel prijs awarded to the Department of Neurology.

**References**
