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Mild congenital muscular dystrophy in two patients with an internally deleted laminin α2-chain

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Congenital muscular dystrophy (CMD) is a group of clinically and genetically heterogeneous disorders inherited in an autosomal recessive mode. The α2-chain of laminin-2 (previously called merosin) has been shown by immunohistochemical and genetic analyses to be implicated in the pathogenesis of the 'classic' form of CMD. In the 'merosin-deficient' subgroup, which represents about half of the cases, more definite evidence of the involvement of the laminin α2-chain has recently been reported with the identification of mutations in the gene encoding the α2-chain of laminin 2 (LAMA2) in CMD patients. Here we report on two siblings from a consanguineous family expressing an internally deleted laminin α2-chain as a result of a splice site mutation in the LAMA2 gene which causes the splicing of exon 25. The predicted protein lacks 63 amino acids in domain IVa which forms a globular structure on the short arm of the α2-chain. Interestingly, these patients appear mildly affected compared to others who completely lack this protein. This situation presents a striking analogy with Becker muscular dystrophy, where in-frame deletions in the dystrophin gene result in the expression of a semi-functional protein and lead to a mild phenotype.

INTRODUCTION

Congenital muscular dystrophy (CMD) is a clinically and genetically heterogeneous group of autosomal recessive neuromuscular disorders of early onset (1–5). In the 'classic' form of CMD, clinical manifestations are limited to skeletal muscle with no clinical involvement of the central nervous system (CNS) although changes in the white matter have been detected by MRI (6–9). The histological changes in muscle biopsies consist of connective tissue proliferation, large variation in the size of the muscle fibers as well as some necrotic and regenerating fibers. Two groups of 'classical' type of CMD cases can be distinguished according to the status of the α2-chain of laminin-2 (formerly merosin; 10) with about half of the cases displaying a deficiency of this protein (5,11–13). However, even these 'merosin-deficient' CMD cases represent a heterogeneous subgroup since some patients display a total deficiency of the α2-chain of laminin-2 whereas this protein is expressed in others, though at a reduced level. Linkage analyses and homozygosity mapping studies have led to the localization of the CMD locus to chromosome 6q2 (14,15), in the region containing the gene encoding the α2-chain of laminin (LAMA-2; 16). Recently, mutations affecting this gene have been identified in CMD patients (17,18).

The expression of the α2-chain of laminin-2 is also altered in the *dystrophia muscularis* (*dy*) mouse (19–22) and its allelic variant (dy^{2J}). A splice mutation affecting the murine gene encoding this protein, localized on chromosome 10 (21) has recently been identified in the dy^{2J} mouse, resulting in the expression of a truncated protein (23,24). Interestingly, the dy^{2J} mouse displays a less severe phenotype than the *dy* mouse (23) which lacks the laminin α2-chain.

Laminins are a family of large extracellular glycoproteins (25) which display a complex and still unclear repertoire of biological functions. Laminin-2, the isoform involved in CMD (11), is specifically expressed in the basal lamina of striated muscle and

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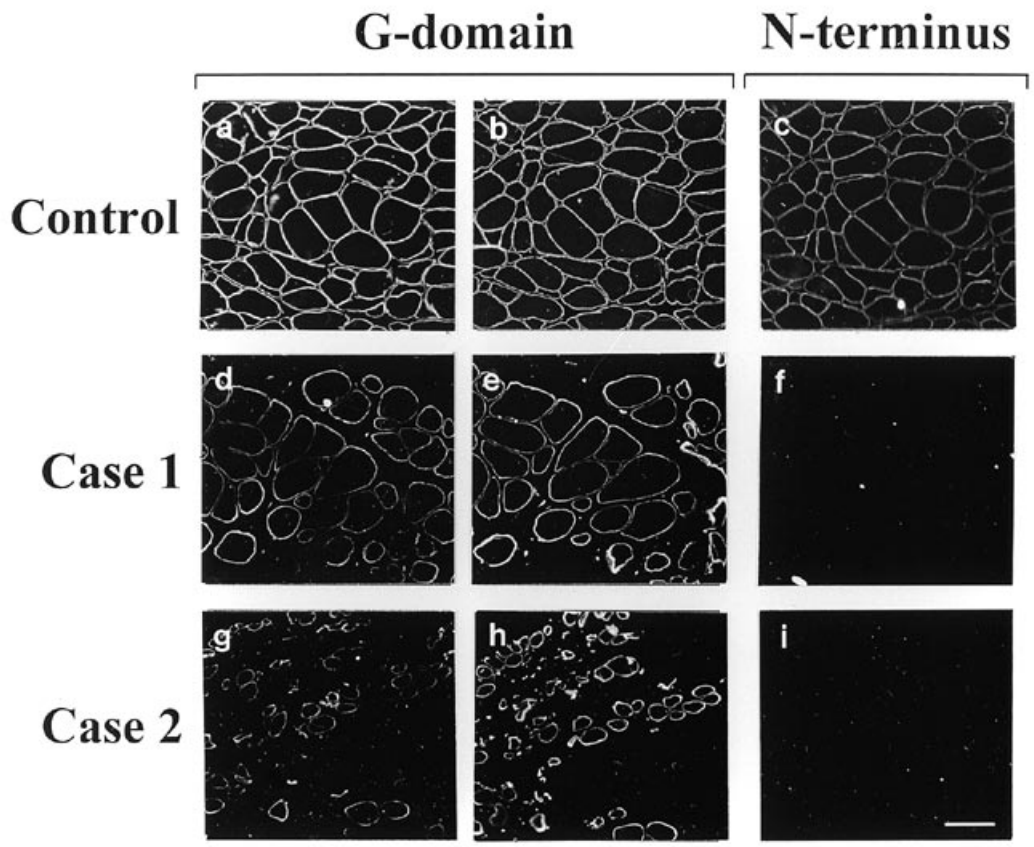

Figure 1. Immunocytochemical analysis of the laminin α2-chain in human skeletal muscle biopsies. Using a monoclonal (5H2; **a**, **d**, **g**) as well as a polyclonal [α2(G); **b**, **e**, **h**] antibody recognizing the G-domain of the protein, a near normal level of expression of the α2-chain is observed in Cases 1 and 2, with a 'patchy' pattern seen on some fibers, as compared to a control individual (Control). However, the use of monoclonal antibody directed against the N-terminal portion of the protein (4H8-2; **c**, **f**, **i**) revealed a more drastic reduction in the expression of the laminin α2-chain in Cases 1 and 2. The horizontal bar corresponds to 50 μm.

peripheral nerve (26–29). As all members of the laminin family, it is composed of three chains: one heavy (α2) and two light chains (β1 and γ1) that assemble in a cross-shaped molecule with three short arms and one long arm (25,30). The C-terminal ends of each chain interact to form the triple stranded long arm of the molecule, stabilized by disulfide bonds, with a large globular (G) domain contributed to by the α2-chain (16,31). The α2-chain of laminin consists of six domains: I and II are part of the long arm; IIIa, IIIb and V contain cysteine-rich EGF-like repeats and are predicted to have rigid rod-like structures; IVa, IVb and VI are predicted to form globular structures (16,31). Laminin α2-chain has been shown to be a native ligand for α-dystroglycan, an extracellular component of the dystrophin-associated glycoprotein complex (DGC). This complex constitutes a link between the subsarcolemmal skeleton and the extracellular matrix (32–34). A number of components of the DGC have now been shown to be involved in muscular dystrophies (35,36) suggesting a crucial role of laminin-2 and the components of the DGC in maintaining the integrity of muscle cell function.

In this study we report the identification of a novel type of mutation in the human LAMA2 gene and causing CMD in two siblings from a consanguineous Saudi Arabian family presenting a slight reduction in the expression of laminin α2-chain. We identified a T→C substitution at position +2 of the consensus donor splice site of exon 25 leading to an aberrant splicing of this exon and resulting in an in-frame deletion of 63 amino acid residues in domain IVa of the laminin α2-chain. This truncation occurs on the short arm of the molecule and may therefore alter the laminin network in muscle basement membranes and, by disrupting the linkage between the extracellular matrix and the DGC, may lead to muscle cell degeneration, as previously suggested by Sunada *et al.* (24) in the case of the *dy*^{2J} mouse.

RESULTS

Immunofluorescence analysis of skeletal muscle sections taken from two siblings from a consanguineous Saudi Arabian family diagnosed with CMD was performed using a monoclonal (27,28) or a polyclonal antibody (see details in Materials and Methods) recognizing the G-domain of the laminin α2-chain. A near normal level of expression of the protein was detected in both patients compared to normal control tissue (Fig. 1). However, a drastic reduction of the staining was seen with a monoclonal antibody raised against the N-terminal region of the protein (37).

Immunoblotting analysis suggested the expression of a truncated protein in these patients since two bands of ~170 and 120 kDa were identified using a polyclonal anti-α2-chain antibody which detected an N-terminal fragment of 300 kDa in normal control skeletal muscle (Fig. 2). These bands likely represented proteolytic fragments of the protein. However, the truncated α2-chain still seemed capable of assembly with chains β1 and γ1 since the heterotrimeric molecule was detected (Fig. 2). Corre-

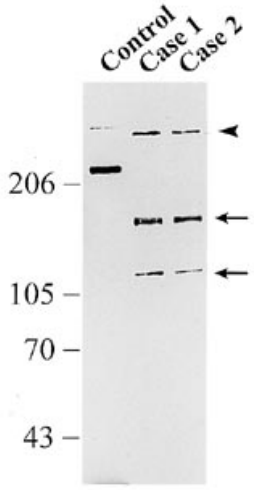

Figure 2. Immunoblot analysis of laminin α2-chain in skeletal muscle EDTA extracts. Staining with a rabbit polyclonal antibody detected the 300 kDa N-terminal fragment of the α2-chain in a control individual (Control) whereas two smaller fragments of ~170 and 120 kDa (arrows) were observed in CMD patients (Case 1 and Case 2). In all three lanes the 700 kDa heterotrimeric laminin-2 molecule was detected (arrowhead). Molecular weights (in kDa) are indicated on the left side of the figure.

spondingly, *in vitro* studies showed that the deletion identified in the dy^{2J} mouse seemed to render the protein more sensitive to proteolysis (P. D. Yurchenco, personal communcation).

Because of the consanguinity existing in this family, we assumed that both affected siblings were likely to be homozygous for the same mutation and thus arbitrarily focused our study on Case 1. RT-PCR amplification was performed using a variety of oligonucleotide primers scattered along the cDNA sequence of the LAMA2 gene. A smaller fragment was obtained for one set of primers as compared to a control individual (Fig. 3a). Direct sequencing of this PCR product revealed a 189 bp in-frame deletion resulting in the loss of 63 amino acids in domain IVa (residues 1246-1308) of the protein (Fig. 3b). Based on the genomic structure of the LAMA2 gene (38), primers surrounding the deletion were designed and used for amplification on DNA of all members of the family. Direct sequencing of the PCR products revealed a T→C substitution at position +2 of the consensus donor splice site of exon 25. This 3973 +2 T→C mutation was found in a homozygous state in both patients (Fig. 3c) and induced the splicing of exon 25 by alternately using the donor splice site of exon 24. As expected, both parents were heterozygous for the mutation (Fig. 3c). The unaffected son of this family did not carry the mutation (Fig. 3c). Ninety-four chromosomes from non-related individuals were also tested for the 3973 +2 T→C mutation by direct sequencing of PCR products but this mutation was never found (data not shown).

DISCUSSION

We have thus identified a novel mutation in the human gene encoding the laminin α2-chain which leads to the expression of an internally deleted protein in two CMD siblings. The results obtained by immunofluorescence analysis with antibodies recognizing different domains of the laminin α2-chain were very interesting since both antibodies recognizing the G-domain of the protein (27,28) detected a near normal level of expression of the protein with only a few fibers showing a 'patchy' pattern. On the contrary, the use of antibodies recognizing the N-terminal region of the protein (37 and data not shown) revealed a more obvious reduction in the expression of the protein, consistent with the deletion in domain IVa of the protein. These results demonstrate that using more than one antibody can provide valuable indications as to what domain(s) of the laminin α2-chain may be affected in CMD patients.

The mutation identified here is similar to that found in the dy^{2J} mouse (23,24), i.e. a nucleotide change in a donor splice site leading to an aberrant splicing mechanism and to the expression of a truncated laminin α2-chain. In both cases presented here, as well as in the dy^{2J} mouse, the in-frame deletion occurred in the short arm of the laminin α2-chain, known to be involved in the formation of a laminin network in basement membranes (39,40). It is therefore likely that, in the dy^{2J} mouse as well as in these patients, the deletion may disrupt the formation of the laminin network and the protein may become more sensitive to proteolysis, as suggested by the immunoblotting analysis.

At the clinical level, it is worth noting that the two CMD cases presented here appear to be less severely affected than the 'merosin-deficient' cases described in the literature (5). Correspondingly, the phenotype of the dy^{2J} mouse is milder than that of the *dy* mouse which presents a drastic reduction in expression of the protein (23). This situation is reminiscent of the Duchenne and Becker muscular dystrophy cases: while Duchenne patients are very severely affected and often display out-of-frame deletions in the dystrophin gene, the mildly affected Becker patients have in-frame deletions in this gene. Indeed, as the number of mutations identified in the human LAMA2 gene increases, it will provide useful information to establish a genotype/phenotype correlation and to refine the clinical definition of this heterogeneous group of muscular dystrophies. Also, in light of the immunofluorescence data presented here, it could be interesting to reevaluate the mildly affected 'merosin-positive' patients with different antibodies.

MATERIALS AND METHODS

Subjects

A 3.5 year old boy (Case 1), was referred to King Khalid University Hospital because of muscle weakness and delay in motor development. He showed muscle hypotonia and could not control his head when pulled to a sitting position after the age of 5 months. Although he could roll over at 5 months and sit independently at 7 months, he never attempted crawling but was ultimately able to walk at 26 months of age. Thereafter, he stumbled easily and had difficulty raising from the sitting position. His younger sister (Case 2) also showed hypotonia since early infancy and had poor head control. She eventually achieved walking at the age of 3 years and 8 months. Both pregnancies had been carried to term and both deliveries had been normal. Both patients are still walking at the present time. In both cases, investigations revealed normal electrocardiogram and nerve conduction but electromyography showed myopathic features. Muscle biopsies from the *vastus lateralis* showed replacement of muscle fibers by fat tissue associated with an increase in endomysial and perimysial connective tissue. There was a wide variation in fiber size. Both atrophic and markedly hypertrophic fibers were observed along with a few hyalin fibers. In addition,

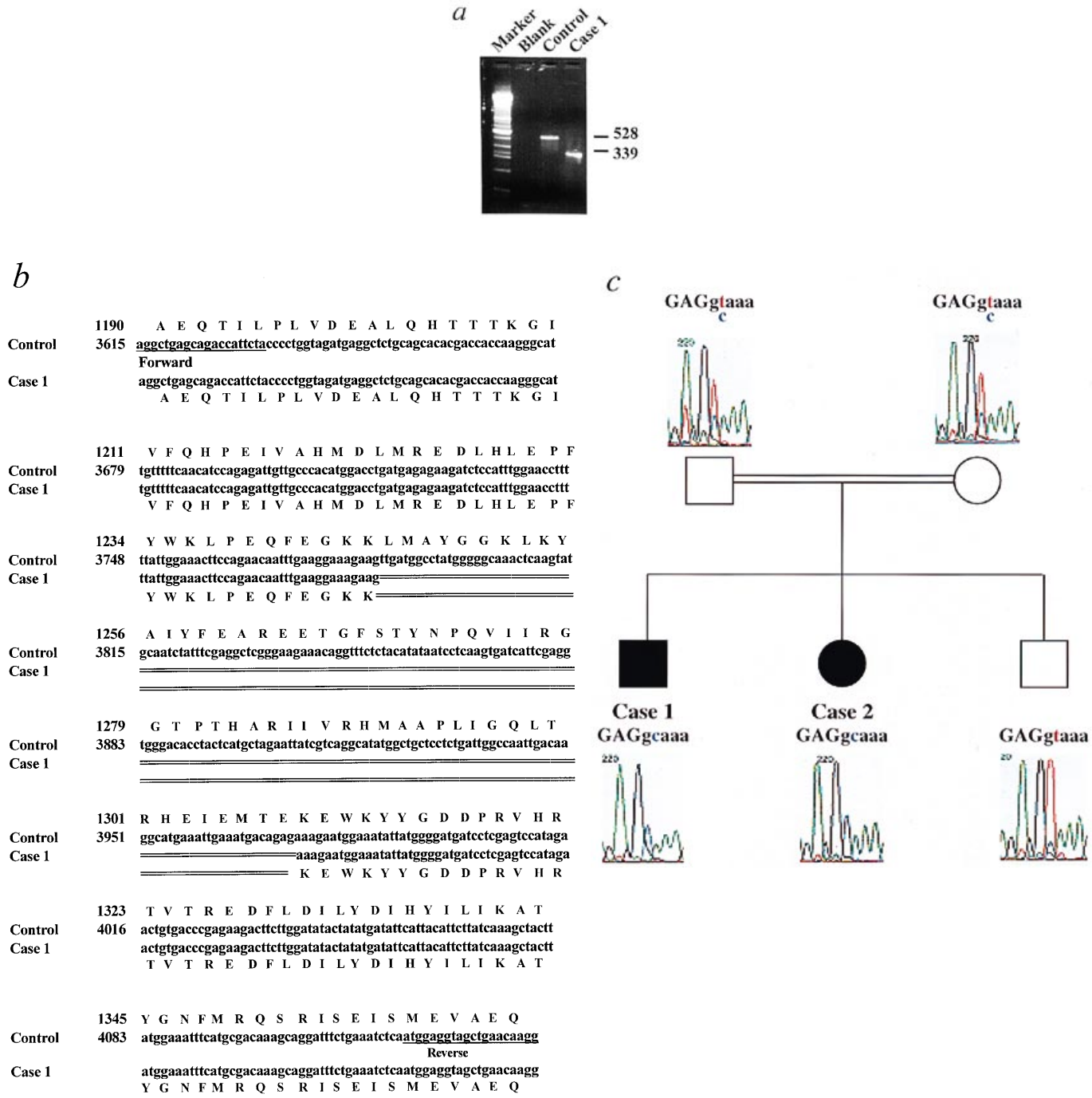

Figure 3. (**a**) Electrophoresis of RT-PCR products. Using primers Forward and Reverse, the fragment obtained from CMD Case 1 was 189 bp smaller than the fragment obtained from a control individual (Control). Lane 1 (Marker) represents a 100 bp DNA ladder. Sizes (in bp) are indicated on the right side of the figure. (**b**) Nucleotide and amino acid sequence comparison of the RT-PCR fragment amplified from Case 1 with a control individual. (**c**) Direct sequencing of PCR products amplified from genomic DNA. Squares and circles represent male and female individuals, respectively. Affected and unaffected individuals are depicted by solid or open symbols, respectively. The horizontal double line indicates the consanguinity between the parents. Exon and intron sequences are written in block capitals and lower case, respectively.

there were degenerating, regenerating and split fibers. Serum creatine kinase levels of Case 1 were elevated (1187 U/L, Control <180 U/L) whereas they were only slightly raised in Case 2 (256 U/L). In both patients, magnetic resonance imaging of the brain showed no evidence of atrophy but increased signal intensities in the white matter on T2-weighted images. The spread of the white matter changes appeared wider in Case 2, with a distribution pattern more pronounced around occipital horns of the lateral ventricles, but also anteriorly around the temporal horns and at the level of the centrum semiovale (data not shown).

Immunohistochemistry

Skeletal muscle cryosections (7 μm thick) were prepared from normal control tissue and CMD Cases 1 and 2 biopsy specimens. Indirect immunofluorescence microscopy was performed as previously described (41). Monoclonal anti-human laminin α2-chain antibody, clone 5H2, recognizing the G-domain of the protein (27,28) was purchased from GIBCO-BRL. A protein containing the last 1444 residues of the human laminin α2-chain was generated by transfecting insect Sf9 cells with the corresponding α2-chain cDNA fragment cloned into pVL1392ss, a modified baculovirus expression vector. The secreted α2(G) protein was purified to homogeneity as described previously(42). Rabbit polyclonal antibodies were generated through successive rounds of immunization using 0.2 mg of protein in complete Freund's Adjuvent. Total IgG fraction was obtained using protein A affinity chromatography. Monoclonal antibody 4H8-2 was raised in rat to native mouse laminin-2 (37). Specificity for laminin α2-chain was demonstrated by reaction with laminin-2, but not laminin-1 in ELISA, immunoprecipitation and Western blots. Cross-reaction with human laminin α2-chain was as previously described (43). The sections were examined under a Zeiss Axioplan fluorescence microscope. Photographs were taken under identical conditions with the same exposure time.

Immunoblotting

Biopsied skeletal muscle cryosections from normal control and CMD Cases 1 and 2 were subjected to the procedure previously described (24) except that the detection was performed using the enhanced chemiluminescence system (ECL kit RPN 2101; Amersham). Rabbit polyclonal anti-laminin α2-chain antibodies were generated as previously described (44) against a murine GST-fusion protein spanning amino acid residues 1682–1884, which are located within domains I+II of the long arm of the laminin α2-chain.

RT-PCR

Total RNA was prepared from biopsy samples using RNAzol (Tel-Test, Friendswood, TX) according to the manufacturer's directions. Single-stranded cDNA was generated by reverse transcription with Stratascript (Stratagene) using an oligo-$(dT)_{18}$ primer. Several primer couples spanning the entire cDNA previously described (45) or designed by us (forward primer 5′-AGGCTGAGCAGACCATTCTA-3′, reverse primer 5′-CCTTGTTCAGCTACCTCCAT-3′ at positions 3615 and 4124 of the cDNA sequence, respectively) were used for amplification. One tenth of the single-stranded cDNA was subjected to PCR amplification in a final volume of 50 μl containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH8.3 and 100 ng of each primer. After 5 min at 94°C, 200 μM of each dNTP and 2.5 U *Taq* polymerase (Boehringer Mannheim) were added, and 35 cycles were carried out as follows: 40 s denaturation at 94°C, 30 s annealing at the appropriate temperature and 30 s extension at 72°C. Five μl of PCR products were analyzed by electrophoresis on 2% Seakem agarose gel stained with ethidium bromide.

Genomic DNA amplification

Genomic DNA (50–100 ng) from each member of the family was used as a template for PCR amplification. Reactions were performed as decribed above. To amplify the region surrounding the deletion, a forward primer designed in an intronic sequence (5′-CACACCATTTGGAGATTTATC-3′) was used in combination with a reverse primer designed in an exonic sequence (5′-TCGGGTCACAGTTCTATGG-3′). Thirty five cycles of amplifications were performed as described above with an annealing temperature of 55°C. Five μl of PCR products were analyzed by electrophoresis on 2% Seakem agarose gel stained with ethidium bromide.

Sequencing

Prior to sequencing, PCR products were purified using the QIAquick PCR purification kit (QIAGEN). DNA sequencing was performed using the dye terminator cycle sequencing chemistry with AmpliTaq™ DNA polymerase, FS enzyme, and was analyzed on a 373A Sretch Fluorescent Automated Sequencer (Applied Biosystems).

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