Biochemical and Biophysical Research Communications **280,** 39–44 (2001)
doi:10.1006/bbrc.2000.4086, available online at http://www.idealibrary.com on IDEAL®

Structural Analysis and Mutation Detection Strategy for the Human LamC3 Gene

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Received November 20, 2000

Laminins are heterotrimeric extracellular matrix molecules, present in a wide range of basement membranes within human tissues. They consist of a combination of different α, β, and γ subunits. Three different γ subunits have been described to date. Two of them, the γ1 and γ2 chains are constituents of basement membrane related laminins, while the γ3 chain was detected in skin, heart, lung, reproductive tract, brain, and in the retina. Unlike other laminins, the expression of the γ3 chain was localized to peripheral nerves and to the apical surface of ciliated epithelial cells and in the retina. To further investigate the function and the possible pathogenic role of laminin γ3 in human disease, we elucidated the structure of the corresponding LAMC3 gene which encodes this polypeptide. Here we report the genomic organization of the LAMC3 gene and a mutation detection strategy for use in genetic studies.

Laminins are heterotrimeric molecules consisting of three polypeptide subunits which are found in a wide variety of tissues, mainly as structural proteins, but also playing a role in signal transduction. The different characteristics and functions of laminin molecules depend on the combination of subunit components. Several different polypeptide subunits have been identified to date. They are distributed into subgroups of α-chains, β-chains, and γ-chains, while the genes encoding these polypeptides are designated LAMA, LAMB, and LAMC genes respectively (1). The molecules have an amino-terminal short arm and a carboxyl-terminal long arm, where the short arm consists of globular domains separated by epidermal growth factor like domains and the long arm consists of heptad repeats (2). Their structural and signal transduction functions are thought to play an important role in the formation and maintenance of basement membranes and their interactions with neighboring structures (3).

A total of three laminin γ-chains have been identified, including the γ1 (LAMC1), γ2 (LAMC2), and γ3 chains (LAMC3). Two of them, the γ1 and γ2 chains, are components of laminin 4 and laminin 5, which comprise important structural components of the basement membrane zone of epithelial tissues (4). The cDNA encoding the γ3 chain was recently described (5). Although this laminin gene contains structurally homologous domains similar to laminin γ1, it seems to play a different role in the body by virtue of distinct expression pattern. While the γ3 chain is present in a number of different tissues, such as skin, eye, heart, lung, reproductive tract and placenta, its localization within the tissues is unusual (Koch *et al.*, 1999; Libby *et al.*, 1999). In the cutaneous basement membrane, γ3 is detectable only at nerve crossings and surrounding the Merkel cells in whisker follicles. In the tissues of the reproductive tract, γ3 was detectable at the base of the cilia on the apical surface of epithelial cells in monkey, bovine and rat (Koch *et al.*, 1999). Examination of rat bronchial epithelia gave similar results, showing that γ3 was detected at the apical surface of the epithelial cells (Koch *et al.*, 1999). Laminin γ3 is expressed in the neural retina at the apical surface of the retina and in the outer synaptic layer (Libby *et al.*, 1999). Two different γ3 containing laminin molecules have also been isolated from the cerebellum, supporting the hypothesis that laminin γ3 is an important component of the central nervous system (Champliaud, Brunken, and Burgeson, unpublished observation). To further investigate the possible role of LAMC3 via elucidating its potential role in diseases, we have elucidated the exon-intron structure of this gene and developed a mutation screening strategy.

0006-291X/01 $35.00

TABLE 1

PCR Primers to Amplify the Coding Sequence of LAMC3

Exon	AT	Forward primer	Reverse primer
E1	55	GCCCCGGCCAGAGCGCG	GTGCCCCCAGCCCGCGC
E2	55	GGGCATTGTCCTTTCCAGTG	GACCCTCCTACTGCCGTGAC
E3	55	ATGGCACCCTCTCCCCTCTG	CACCCCACAGCAGCCACCTT
E4	55	CCTCCTCTGTGTCTTTTCCC	CTCCCCTTCTGCTAGTCCTT
E5–6	55	ACCATGCTGTTGCCCTTCTC	AGACAGGAAGGGATGGGGAG
E7	55	TGTTGCCATTCTGAATTTGC	GCTTTGGTGCCTCATCAGTG
E8	55	CCCCTTCCTGGCTGATTCAC	CCATCATCTCACCCCCGACC
E9	55	ACTGATCGGGGGGTGTCTGT	CAGCAGGAGGAACCGACACA
E10	55	GATTTTGATTTCCTACCCAC	CTCAGACAGAAGGGGATACT
E11	55	GGGATCTCACTTTGACCGCT	GTCTTGGGCATGTGGTTGTC
E12	55	GCTTGTGCCTCCTCTCTTCC	TACTCATCCCGCTGTTTTGG
E13	57	TGCTGCCTCTGCCTGGG	CCCCGGGCAGGAGTCAT
E14	61	TTCACACCCACCCTCATCCC	CCAGGGCAGAAGAGAAGGGG
E15	55	TCCCAACACCCCTTCCTGAT	CCCGATGCCTTCTTCCCTAC
E16	55	CCTCAGACCCAGTTCCTTCC	CCATCAGTCATCTCTGCCCA
E17	55	CTTAGGGCATTCTGGGAACA	CTGGCACTTGCTGGGAATAC
E18	61	AGCGGGAGTGTCTGGGCAGG	CTCCCCTTGCCTCCCACCCT
E19	55	TGCCTTTGGGACTACCTGTT	AACATCCTCTCGCTGAATCC
E20	55	CTCCGCTCACTGAATGCCGA	GGCACCTGTGGGGCTGACTC
E21	55	AACAGAAACAGGGCATGGAG	GAACGTCAGGACTCAAGGGA
E22	61	GACAGGGATGGGGAGGGCTA	CGACCCCACACGGAGNTNTG
E23	61	GGNTTGGGTTCTTGGCTGGC	CCTCCTGCAACCCTGACCCT
E24	55	ATTTGGTGATGATTTGGGCA	GGGCGTTCGTCATTACACAG
E25	55	TTCAGACCTCCACTCGTTGT	GGAGTAGTCAGCACGGATGT
E26	51	TTCTTCTCCCTGCCACTGCC	CAGGGGCAGGAGGCGATAGG
E27	55	CCCTACCCTATCGCCTCCTG	GGCAATGAGGACAAGGAGGA
E28	63	AGGCTNCAGGGGCTGGGAGG	CAGGTGGGGGAGTGTGTGCC

Note. The table contains the PCR primer sets used to amplify the coding sequence of the LAMC3 gene. AT indicates the annealing temperature used for the primer set. While one primer set was usually used to amplify one exon and the flanking intron sequences, because of the small size of intron 5 we amplified exons 5 and 6 together in one PCR reaction.

MATERIALS AND METHODS

Determination of Exon-Intron Borders of LAMC3

To identify the exon-intron boundaries of the LAMC3 gene, oligonucleotide primer pairs were designed based on the cDNA sequence, in locations based on the exon-intron organizations of LAMC1 and LAMC2. The forward and reverse primers were placed at a distance of 50–100 bp from the presumed exon borders. Each primer pair was optimized using the program Oligo Primer Analysis Software (National Biosciences, Inc., Version 4.0). The oligomer primers were 20 nucleotides in length, contained minimal secondary structure, between 45 and 65% G/C content, and were free of the potential for primer-dimer formation. Primers were synthesized using an automated oligonucleotide synthesizer (Applied Biosystems).

For PCR amplification, ~500 ng of human genomic DNA was used as a template, and the amplification conditions were: 96°C for 10 min, followed by 95°C for 45 s; annealing for 45 s; 72°C for 1 min, for 40 cycles, in an OmniGene thermal cycler (Hybaid, Inc.). Alternatively, in some experiments we used bacteriophage P1 DNA isolated from commercially available clones containing large fragments of the human LAMC3 gene, as a template for PCR amplification (Research Genetics). The annealing temperature was individually determined for each set of primers. Amplification buffer contained 1.5 mM $MgCl_2$ and 2 U of AmpliTaq Gold DNA polymerase (Perkin Elmer), in a total volume of 50 μl. Aliquots of 5 μl were analyzed on 2% agarose gel electrophoresis. Samples that generated a distinct PCR product on agarose gel electrophoresis were directly sequenced in both directions using the ABI Prism 310 automated sequencing system (Applied Biosystems, Inc.). Exon-intron boundaries were determined by comparison of the cDNA and genomic sequences.

Radiation Hybrid Mapping

The Stanford G3 and Genebridge 4 Radiation Hybrid Mapping Panels (Research Genetics, Huntsville, AL) were screened by PCR with primer pairs derived from each end of the gene, according to manufacturer's protocols. The 5′ end of the γ3 gene, corresponding to exon 2, was amplified using the following primer pair: MF128, 5′-GCT TAT GAG ATC ACG TAT GTG AG-3′; MF155, 5′-CTG CAG CCC AGG GCT CTC CTC GAA-3′. The 3′ end of the γ3 gene, corresponding to exon 28, was amplified with the following primers: MF143, 5′-TGG GTC ATA CAC ACA GAC ATG CAC-3′; MF144, 5′-TCC CAG CAG CAG GAG CTG CAG ATC-3′.

Mutation Detection Strategy

Primer design and PCR amplification for mutation detection. For amplification of coding sequences of LAMC3, oligonucleotide primers were designed for each exon on the basis of flanking intronic sequences in order to generate PCR products containing the exon and parts of the adjacent intron sequences. Primer design and synthesis were performed as described above. For PCR amplification, we used ~500 ng genomic DNA, isolated from peripheral blood lymphocytes according to standard techniques (Sambrook *et al.,* 1989). PCR conditions were identical to those described above. Optimal annealing temperature was determined individually for each set of primers (Table 1).

TABLE 2

Exon-Intron Borders of LAMC3

Exon	Splice donor		Splice acceptor	
1			CTCCGCCTAG	gtaagcgcgggctggggg
2	gggccccttttcctggctgcag	GGAAGGCTTA	TGGGCTGCAG	gtcagggaggagcggggc
3	caccctctcccctctgccccag	GAGTGGGTCA	TGGGCGGCAG	gtaggagggaggagggag
4	cctcctctgtgtcttttcccag	GTGCAAGTGC	GAGTGTCTGC	gtgagtgtctgagtgtca
5	tttccccactcctgccacatag	CCTGCAACTG	CAGTCGGCAG	gtgagtggactcctgatc
6	ttgccctttcctctccccaaag	GCTCCCTACA	GAGGCTGCAG	gtgatgggcgatggggcg
7	tcttgtcctgtctcattggcag	ACCCTGCACT	TATGTGACAG	gtttgtgagctaatccag
8	gcatgtcctgcctccatttcag	ATGTCGCCCG	TTCCACCAGG	gtaagagatgctccctgc
9	ggtgtctgtgtttgctgtctag	GAGCCGAAGG	ACAGCACCAG	gtacctccagcaccagag
10	agccttcttctcttgtcttcag	GGAAGTTCCT	TCAGGTTCCA	gtaagtatccccttctgt
11	ctttgaccgcttctctcgccag	CCTGCAGGAG	AGCCCTGCCG	gtcagtaaagacaaccac
12	tctctcccttctcgctctgcag	GTCCAGTGTT	CCCAACACAG	gtgagtctcctggcaccc
13	tggtgcttgtcttgcccctcag	GGATCTGTGT	GGCCAGAGAG	gtaatgactcctgcccg
14	cacgtgctccctatacacacag	GGCGGCGCTG	AAATGCATGC	gtgagtacctacctccag
15	cccttcctgatcctgcccccag	CTTGCAGCTG	GCTGCCGGAG	gtaggtagggtgagactg
16	cctgttccttccctgatcacag	CTGCAAGTGT	GGCTGCCGGG	gtaaggaggctgggtcct
17	gcactgcccctggcccctctag	CCTGCAGGTG	GAAGGAGGAG	gtgagtcggccagaccac
18	cttttcttcctgtcctctccag	GCAGCCAAGC	CTGCTTCCAG	gtacagcaggagcgcaga
19	ctgggctgtgggcttcccatag	GGGCTCGGGA	CGCGTCTCTG	gtatcccaggggacccccc
20	cctcttcctcttctttctacag	GAGATTCCTC	TCGCCAGGAG	gtgatgtccaagacatgg
21	caggtgggtgtccgtcacacag	CCACAGAGAC	TGGAGGACAG	gtgaggcctccccaggtg
22	cgtggcctctgcttcctcccag	GTACCAGGAG	GGGAGCTCTG	gtcagctcatttgtctca
23	tgagcagattgtctccctgcag	CCTCAGAAGT	CCTCACAATG	gtcagctcttgtgcttt
24	gcctctcctcatctctctccag	CTGCACCAGG	GATCTGGAAG	gtacgtgagtccagctga
25	ccagcctcagccgggtttgcag	GAATGAAGCT	CAGTGCCAAG	gtcaggggtggtggctgt
26	gccactgccacccatcccatag	CTTGCCAAGG	AGCTGAGCGG	gtacgtttgcagggccct
27	tcttcctcctcccccctggaaag	GTGGGTGCTG	GCCAGGCTGG	gtaggggggcctaaggctg
28	ggctgtttgtgccccaccacag	GGTCGCTGGA		

Note. The table demonstrates the exon-intron boundaries in the LAMC3 gene, organized according to exons. The 5′ and 3′ parts of the exon sequences are in capitals.

Heteroduplex Analysis

To test the primer pairs, we used genomic DNA from unrelated individuals from different geographic locations (North America, Eastern Europe, Far East) as a template for PCR amplification. Eight microliters of the PCR products was prepared for heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE). CSGE was performed in a vertical gel containing 10% polyacrylamide (99:1 Acrylamide: 1,4-Bis (acryoyl) piperazine), 15% ethylene glycol, 10% formamide and 0.5× TTE buffer, pH 9.0 (Tris Taurine EDTA buffer, US Biochemicals), with sample preparation consisting of denaturation at 98°C for 8 min followed by a 30 min incubation at 68°C before loading. Heteroduplexes were visualized by staining with ethidium bromide. Bands of altered mobility detected in heteroduplex analysis were directly sequenced using the ABI Prism 310 automated sequencing system (Applied Biosystems, Inc.).

RESULTS

Exon Intron Borders

We determined the complete exon-intron structure of the LAMC3 gene. Comparison of the previously published cDNA with the genomic sequences revealed a total 28 exons (Table 2). The translation initiation codon was present in exon 1, while the translation termination codon was found in exon 28.

Radiation Hybrid Mapping

MF128-155 was linked to marker SHGC-4319 in the Stanford G3 panel, with a lod score of 10.39 and a distance of 0. MF143-144 was linked to marker SHGC-4319 in the Stanford G3 panel, with a lod score of 6.84 and a distance of 17 cR. MF143-144 was linked to marker WI-6494 at a distance of 2.84 cR on the Genebridge 4 Whitehead framework map. In addition, an STS marker derived from the γ-3 sequence was found in the GeneMap98 database. The STS marker WI-14302 (GenBank Accession No. G22528) is derived from an EST which represents the 3′-most exon of the LAMC3 gene. Based upon comparisons of the SHGC and WI RH maps, the LAMC3 gene is probably organized with the promoter toward the telomere and the 3′ end towards the centromere of chormosome 9 at 9q31-34.

Mutation Detection Strategy

Screening of PCR products from unrelated, unaffected individuals from different geographic locations resulted in the detection of several bands of altered mobility. Sequencing the corresponding PCR products,

TABLE 3

Sequence Variants Detected in the Coding Sequence of LAMC3

Exon	Nucleotide	Amino acid
3	789 G-to-C	Ser-to-Thr
3	83 G-to-A	Val-to-Val
4	952 A-to-G	Ala-to-Ala
6	1315 A-to-G	Thr-to-Thr
9	1661 T-to-G	Ser-to-Ala
10	1728 G-to-A	Gly-to-Glu
11	2029 C-to-T	Ser-to-Ser
14	2656 C-to-T	Ser-to-Ser
19	3341 G-to-A	Gly-to-Ser
21	3604 C-to-T	Thr-to-Thr

Note. The table lists the sequence variants we detected in the LAMC3 gene while screening clinically unaffected, unrelated individuals. The first column shows number of the exon, in which the variant was found, the second column shows the nucleotide change according to the published cDNA sequence of LAMC3 (GenBank Accession No. NM_006059, (5)). The third column demonstrates the result of the sequence variant at the protein level.

we were able to demonstrate the existence of different sequence variants. We found single nucleotide polymorphisms that did not result in an amino acid changes in exons 3, 4, 6, 10, 11, 14, 21. We found single nucleotide polymorphisms the did result in single amino acid changes in exons 3, 9, 19 (Table 3). In addition to the sequence variants detected in the coding sequence of LAMC3, we found one variant in a noncoding region. The 3′ region of intron 16 consisted of tandem 31 bp repeats, immediately preceding the last nucleotide of the intron. Approximately 50% of the individuals we tested carried 3 tandem 31 bp repeats on one allele and 4 tandem repeats on the other allele (Fig. 1). The remainder of the individuals were homozygous for either one of the variants, in an approximately equal ratio. These sequence variants were observed in unrelated, unaffected individuals, in some occasions from different geographic regions, in both the homozygous and heterozygous states; therefore, we concluded that they are polymorphisms.

TABLE 4

Comparison of Exon-Intron Organization of the LAMC Genes

Exon	LAMC1	LAMC2	LAMC3
E1	418 + UTR	79 + UTR	373 + UTR
E2	305		305
E3	131		131
E4	167		167
E5	189	189	189
E6	118	136	118
E7	99	99	99
E8	137	137	137
E9	123	123	180
E10	190	190	194
E11	113	113	114
E12	222	219	118
E13	189	183	189
E14	246	246	246
E15	154		154
E16	143	143	143
E17	179		179
E18	157	157	143
E19	206	206	206
E20	80	80	76
E21	138	156	135
E22	145	145	149
E23	150	153	150
E24	115	115	103
E25	200	200	200
E26	159	159	150
E27	100	100	100
E28	254 + UTR	105 + UTR	248 + UTR

Note. The table compares the exon structure of the genes encoding the laminin γ chains. The exons are listed according to the exon numbering of LAMC1, the table shows the exon sizes in basepairs.

DISCUSSION

In this study, we present the complete exon-intron organization of the human laminin γ3 gene. The overall structure of LAMC3 is most similar to the LAMC1 gene. The homologues of LAMC1/LAMC3 exons 2–4, 15, and 17 are not present in LAMC2, but throughout the rest of the gene, LAMC2 also shows a significant homology to LAMC1 and LAMC3 (Table 4, Fig. 2).

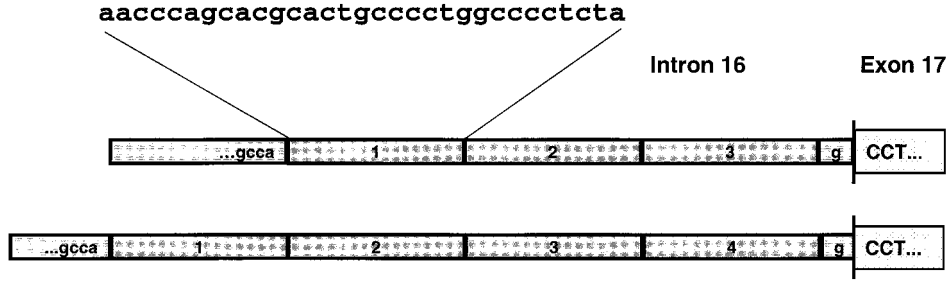

FIG. 1. Tandem repeat polymorphism in intron 16 of the LAMC3 gene. The figure shows the 31-bp tandem intronic sequence polymorphism. The blocks with plain shading demonstrate the 31-bp sequence, repeated three or four times. The blocks with horizontal pattern represent the remaining portion of intron 16 and the blocks with dotted pattern correspond to exon 17.

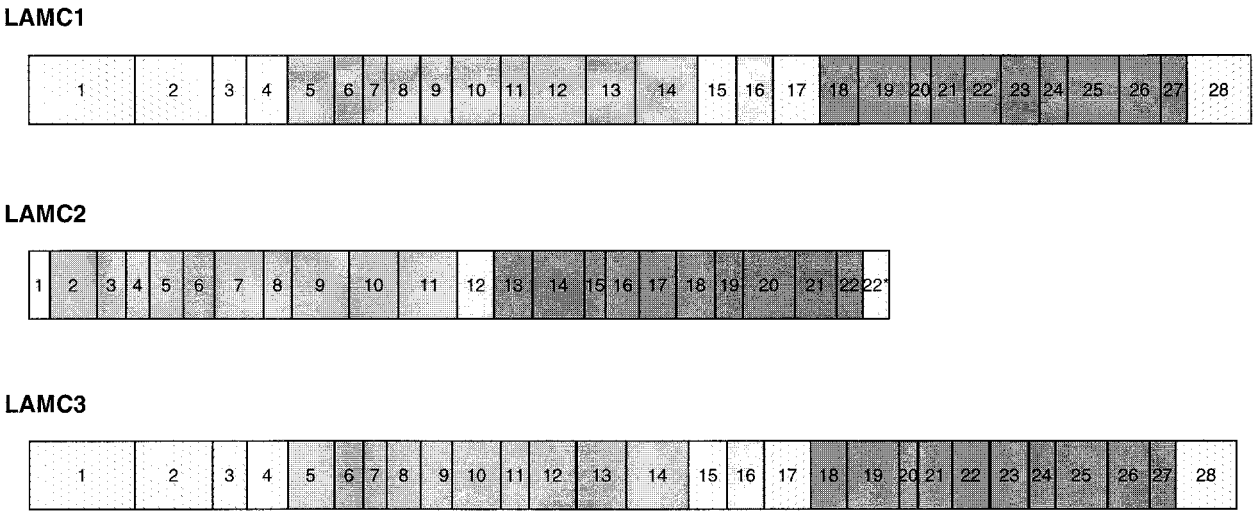

FIG. 2. Comparison of the exon arrangement of the LAMC genes. The diagram demonstrates the arrangement of exons with identical or highly similar size (plain shading). Exons showing significant size difference are marked with dotted pattern. Groups of corresponding exons marked with the same shading in the 3 genes. Note the high resemblance between LAMC1 and LAMC3.

Despite the strong structural homology, the LAMC3 gene demonstrates an expression pattern different from the LAMC1 and LAMC2 genes (4–6). Previous expression studies localized laminin $\gamma 3$ in several human tissues. In particular, the expression of LAMC3 in ciliated epithelial apical surfaces such as in the retina, male and female reproductive tract and lung and expression in peripheral nerve endings, raised several possible candidate human disorders for mutations in this gene.

One candidate disease would appear to be deafness with vestibular dysfunction, based on both mapping data (7) and functional considerations (8). A mouse disorder known as whirler deafness, which presents with deafness and vestibular dysfunction in the adult mouse and manifest as head-bobbing and circling behavior, is a likely mouse model for similar human disorders. The syndrome thought to be a result of defects in the membranous labyrinth and the organ of Corti. In both of these organs, ciliated cells and nerve structures are critical for proper function. LAMC3 could be a valid candidate gene for this disorder, as its product, the $\gamma 3$ chain, is expressed in both the sensory structures of the ear and the nerves innervating them (Brunken and Burgeson, unpublished observation). The possible role of LAMC3 in the whirler mouse is further supported by linkage analysis data. The whirler phenotype shows a recessive pattern of inheritance in mouse. Mapping positioned the whirler region on mouse chromosome 4 between the aminolevulinate dehydratase (Lv) and hexabrachion (Hxb) genes (7). Based on the chromosomal location of the human homologues of these genes (ALAD and HXB), the syntenic region in human was predicted to be 9q32–34, which corresponds to the position of LAMC3 (9q31–34) according to our radiation hybrid mapping results, as well as previously reported FISH data (Koch *et al.*, 1999).

Other diseases in which LAMC3 mutation may play a role can be selected on the basis of either chromosomal localization or predicted pathomechanism. Walker-Warburg syndrome (OMIM:236670), a recessively inherited disorder characterized by markedly disorganized cytoarchitecture, lack of lamination, numerous glial heterotopias, and encephalocele, in some cases is mapped to 9q31 (9). Fukuyama muscular distrophy (OMIM: 253800), characterized by cerebral and cerebellar micropolygyria, fibroglial proliferation of the leptomeninges, hydrocephalus, focal interhemispheric fusion, and hypoplasia of the corticospinal tracts is also mapped to 9q31 (10). The clinical characterization of these conditions is difficult due to the complexity of symptoms, therefore, the existence of endophenotypes is a strong possibility and may help to refine diagnosis in both diseases. Therefore, even though retrotransposon insertion in the fukutin gene has been reported to underlie about 85% of all Fukuyama cases (11), due to the suggestive linkage data and the expression of LAMC3 in the brain which is the primary focus of pathology in both cases, the pathogenetic role of LAMC3 mutations in the remaining cases of Fukuyama disease and Walker-Warburg syndrome should be investigated.

In this study, we present a mutation screening strategy based on PCR and heteroduplex analysis, that enables us to screen for mutations in candidate diseases. We tested our screening strategy on genomic DNA samples from unrelated healthy individuals and identified several sequence variants. Most of these sequence variants do not result in an amino acid substi-

tution, and all have been found in unrelated, clinically unaffected individuals. The previously published expression data demonstrate that LAMC3 is widely expressed; therefore, we predict that any significant impairment of the protein function could be related to clinical abnormalities.

Currently, laminin mutations have been shown to underlie junctional epidermolysis bullosa (12). Animal studies also provided insights into possible pathogenetic roles of different laminin molecules (13, 14), but due to the widespread expression of laminins deleterious mutations in most of the laminin molecules may not allow the development of a viable embryo, therefore those mutations would never lead to a disease, which could explain the relatively small number of human disorders successfully associated with mutations in the laminin genes. This study should facilitate the rapid identification of LAMC3 mutations in a candidate human disorder.

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