Electron Microscopic and Immunohistochemical Findings of the Epidermal Basement Membrane in Two Families with Nail-patella Syndrome

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Nail-patella syndrome is an autosomal dominant disorder characterized by nail dysplasia and skeletal anomaly. Some patients have been shown to have ultrastructural abnormalities of the glomerular basement membrane that result in nephrosis. However, little has been reported on the epidermal basement membrane in this condition. This paper reports 2 families with nail-patella syndrome. Direct sequencing analysis of LMX1B revealed that family 1 and family 2 were heterozygous for the mutations c.140-1G>C and c.326+1G>C, respectively. To evaluate the epidermal basement membrane zone, ultrastructural and immunohistochemical analyses were performed using skin specimens obtained from the dorsal thumb. Electron microscopy showed intact hemidesmosomes, lamina lucida, lamina densa, and anchoring fibrils. Immunofluorescence studies with antibodies against components of the epidermal basement membrane zone revealed a normal expression pattern among the components, including type IV collagen. These data suggest that nail dysplasia in patients with nail-patella syndrome is not caused by structural abnormalities of the epidermal basement membrane.

Key words: epidermal basement membrane; glomerular basement membrane; hereditary osteo-onychodysplasia; LIM-homeodomain protein; LIM-homeobox transcription factor 1B; LMX1B; type IV collagen.

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Nail-patella syndrome (NPS; OMIM #161200) is characterized by nail dysplasia and skeletal anomalies, such as aplastic or hypoplastic patella, elbow abnormalities, and iliac horn (1). The disease is an autosomal dominant disorder caused by a heterozygous loss-of-function mutation in the LMX1B gene (OMIM #602575), encoding a member of the LIM homeobox transcription factor 1B, LMX1B (2). LMX1B functions as a transcription factor and is essential for the normal development of dorsal limb structures, the glomerular basement membrane (GBM), anterior segment of the eye, and dopaminergic and serotonergic neurones (3–7). Patients with NPS sometimes have nephrosis-associated renal disease (1). LMX1B regulates the expressions of type IV collagen (COL4) α3 and α4 (α3(IV) and α4(IV)) chains required for normal morphogenesis of GBM (4). Kidneys of some patients with NPS, therefore, show ultrastructural abnormalities including focal or diffuse irregular thickening of GBM with occasional regions of membrane discontinuity (“moth-eaten” appearance) (8, 9). However, little is known about the epidermal basement membrane (EBM). This study evaluated 2 families with NPS who were heterozygous for the splice-site mutations in LMX1B. We analysed the EBM in these families with NPS using electron microscopic and immunohistochemical techniques.

MATERIALS AND METHODS
The medical ethics committee of Hokkaido University approved all the studies described herein. The study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from the participants and/or their parents before the study procedures were conducted.

Patients
Two families with NPS were enrolled in this study. Family 1 was referred to us with nail deformities since birth. The affected family members were a mother (I-2), 9-year-old boy (II-1), 7-year-old twin sisters (II-2 and II-3), and 7-month-old younger brother (II-4) (Fig. 1a). Their thumbnails exhibited dysplasia (Fig. 1b–e). They also had triangular lunulae on their index, middle and ring fingers. The creases of the skin overlying the distal interphalangeal joints of their fingers were missing (Fig. 1f).

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In family 2, the proband was a 6-month-old boy. There was no family history of nail deformity (Fig. 1g). Clinically, he showed thumbnail dysplasia (Fig. 1h). Triangular lunulae and spoon nail were observed on his index, middle, ring, and little fingers. The creases of the skin overlying the distal interphalangeal joints of his fingers were missing (Fig. 1i).

**Genetic analysis**

LMX1B mutation search was performed as follows. Briefly, genomic DNA (gDNA) isolated from peripheral blood was subjected to PCR amplification, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyser (Applied Biosystems, Framingham, MA, USA). Oligonucleotide primers were designed using a website program (http://www.bioinformatics.nl/cgi-bin/ primer3plus/primer3plus.cgi). The entire coding regions of LMX1B, including the exon/intron boundaries, were sequenced using gDNA samples from patients and their family members. For normal controls, 50 healthy, ethnically matched individuals (100 normal alleles) were studied.

**RNA isolation and reverse transcription-PCR**

Total RNA was isolated from the dorsal thumb skin of the affected mother (I-2) of family 1 and the proband of family 2 using the RNasy Mini Kit (QIAGEN, Hilden, Germany) and treated with DNase (Invitrogen, Carlsbad, CA, USA) to remove gDNA. Complementary DNA (cDNA) was synthesized using 3 μg RNA by the SuperScript IV reverse transcriptase (RT) (Invitrogen) and oligo-dT primer (Invitrogen) according to the manufacturer’s instructions. Using the first-strand cDNA as a template, the LMX1B cDNA was amplified using primers that were designed in exon 1 (5’-ATAGCAACAGGTCCGAGTC-3’) and exons 5–6 (5’-GCCAGCTTCTTCATCTTTGC-3’) of the LMX1B cDNA. The PCR products were TA-cloned into the pCRII-TOPO vector (Invitrogen), and then each clone was sequenced separately.

**Electron microscopy**

Skin biopsy samples were fixed with 5% glutaraldehyde in 0.1M cacodylate buffer, followed by post-fixation with 1% osmium tetroxide in 0.1M cacodylate buffer. After dehydration steps in a graded ethanol series, the samples were embedded in TAAB EPON 812 resin (TAAB, Aldermaston, UK). Ultrathin sections (60–70 nm) were stained with uranyl acetate and lead citrate (10) and examined by JEM1400 transmission electron microscopy (JEOL Ltd, Akishima, Tokyo, Japan) at 80 kV.

**Immunofluorescence analysis**

Immunofluorescence analysis was performed using skin specimens obtained from the dorsal thumb of the affected mother (I-2) of family 1 and the proband of family 2. Fresh skin specimens were embedded in an optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and quickly frozen with dry ice. Five-μm cryostat sections were incubated with primary antibodies overnight at 4°C. After washing in phosphate-buffered saline, the sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate (FITC) for 1 h at room temperature. All the stained samples were observed using a confocal laser scanning microscope (Fluoview FV1000, Olympus Optical Co. Ltd, Tokyo, Japan).
Antibodies

The following antibodies against basement membrane zone components were used: monoclonal antibodies (mAbs) GoH3 and 3E1 (Chemicon International, Temecula, CA, USA) against α6 and β4 integrin subunits, respectively; mAb GB3 (Sera-lab, Sussex, UK) against laminin γ2 chain (11); mAb LH 7.2 (Sigma-Aldrich, St Louis, MO, USA) against type VII collagen; polyclonal Ab S1193 against dystonin (BPAG1); C17-C1 against type XVII collagen (12); and HD1-121 against plectin. S1193 and HD1-121 were generously donated by Professor J. R. Stanley of the University of Pennsylvania, USA and Professor Owaribe of Nagoya University, Japan, respectively. For COL4, PHM-12+CIV22 (Thermo Fisher Scientific, Rockford, IL, USA), Texas Red-conjugated H25 (Shigei Medical Research Institute, Okayama, Japan), FITC-conjugated H53+B51 (Shigei Medical Research Institute), H31 (Chondrex, Redmond, WA, USA), and H43 (Chondrex) were used.

RESULTS

Detection of novel splice-site mutations in LMX1B

Direct sequencing analysis of exons and intron-exon boundaries of LMX1B revealed that family 1 and family 2 were heterozygous for the mutations c.140-1G>C and c.326+1G>C, respectively (RefSeq: NM_001174146.1) (Fig. 1j, k). Moreover, we confirmed that the splice-site mutations were absent in the healthy family members, as well as in the 50 ethnically matched control individuals and Exome Aggregation Consortium (exac.broadinstitute.org).

Exon skipping with frameshift resulting from splice-site mutations

Since c.140-1G>C and c.326+1G>C were located at the exon-intron boundaries, we performed splice variant analysis using RT-PCR of cDNA extracted from the dorsal thumb skin of the affected mother (I-2) of family 1 and the proband of family 2. The cDNA was amplified using primers designated in exon 1 and exons 5–6, which showed 2 transcripts, 827 bp (transcript 1) and 640 bp (transcript 2), in family 1 and family 2 (Fig. 1l). Cloning and direct sequencing of the amplified fragments revealed that transcript 1 was wild-type. In transcript 2, exon 2 was skipped and exon 1 was directly connected to exon 3, resulting in a frameshift and a subsequent premature termination codon (p.Asp49Serfs*17) (Fig. 1l).

Normal ultrastructural appearance of the epidermal basement membrane zone

Samples were obtained from the dorsal thumb skin of the affected mother (I-2) of family 1 and the proband of family 2 because the thumbnail deformity was the most prominent. Electron microscopy revealed no remarkable thickening and redundancy of the EBM, which were previously detected in a nail-patella syndrome patient (13). Tonofilament accumulation in the basal cells and disrupted and discontinuous lamina densa, which were detected in the gingiva or skin of Alport syndrome, were not observed in our patient skin (14, 15). The other complicated structures including the lamina lucida, hemidesmosomes, and anchoring fibrils, were intact (Fig. 2a, b).
Protein expression patterns of the epidermal basement membrane zone

Extensive immunofluorescence analysis of the skin specimens from the dorsal thumb of the affected mother (I-2) of family 1 and the proband of family 2 was performed using several antibodies that react with molecules of the dermo-epidermal junction (DEJ). Immunostaining for α6 and β4 integrin subunits, laminin 332, type VII collagen, dystonin, type XVII collagen, and plectin revealed normal DEJ labelling patterns (Fig. 2c–p).

We used 5 antibodies: PHM-12+CIV22 (α1(IV) and/or α2(IV) (epitopes were not determined)), H25 (α2(IV)), H31 (α3(IV)), H43 (α4(IV)), and H53+B51 (α5(IV)), respectively, to assess COL4 expression patterns in the skin specimens. As previously reported (16), EBM contained the [α1(IV)]/α2(IV) and [α5(IV)]/α6(IV) molecules, but not the α3(IV)/α4(IV)/α5(IV) molecules. There was no difference between the patients with NPS and healthy human controls in the expression levels of COL4 subunits (Fig. 3).

DISCUSSION

The EBM is a complex assembly of proteins that play a key role in dermal–epidermal adhesion and regulates many important processes, such as development and wound healing. COL4 is a main structural component of basement membranes and there are 6 different α chains: α1(IV) – α6 (IV) (17). COL4 forms 3 types of triple-helical heterotrimers consisting of the 2 α1(IV)
and 1 α2(IV) chains; 1 α3(IV), 1 α4(IV) and 1 α5(IV) chains; and 2 α5(IV) and 1 α6(IV) chains (18). Two heterotrimeric molecules are detected in GBM of mature mammals; the [α1(IV)/2/a2(IV) and α3(IV)/α4(IV)/α5(IV) molecules are distributed in the sub-endothelial and sub-epithelial layers of GBM, respectively (19, 20). Congenital anomalies of the α3(IV)/α4(IV)/α5(IV) molecules are associated with defects in molecular filtration resulting in Alport syndrome (21). Some patients with NPS have renal diseases, such as nephropathy characterized by ultrastructural abnormalities of GBM (2, 9). Lmx1b knockout mice have strongly decreased expressions of the α3(IV) and α4(IV) chains in GBM (4). In addition, LMX1B binds to a putative enhancer sequence in intron 1 of both mouse and human COL4A4 and upregulates reporter constructs containing this enhancer-like sequence (4). These data indicate that LMX1B regulates the expressions of α3(IV) and α4(IV), the main components of the subepithelial layers of GBM, and that its dysregulation leads to the morphological abnormalities of GBM and nephrosis in NPS (4).

The EBM zone is composed of various complexes (22). Hemidesmosomes consist of plectin, dystonin, α6β4 integrin and type XVII collagen. Lamina densa and anchoring fibrils comprise COL4 and type VII collagen, respectively. Each of the components plays a crucial role in DEJ adhesion and regulates development and wound healing (23). Two heterotrimeric COL4 molecules, [α1(IV)/α2(IV) and [α5(IV)/α6(IV), are detected in the EBM zone (16). Since abnormalities of the EBM zone lead to nail deformities which are also observed in patients with dystrophic epidermolysis bullosa (24), we evaluated EBM in patients with NPS. Electron microscopy showed intact hemidesmosomes, lamina lucida, lamina densa, and anchoring fibrils. Immunofluorescence studies with antibodies against components of the EBM zone revealed normal expression patterns among them. Moreover, in order to analyse COL4 in more detail, we performed an immunofluorescence study using several COL4 monoclonal antibodies against α1(IV) and/or α2(IV), α3(IV), α4(IV), and α5(IV), and the results revealed that there was no distinct difference between patients with NPS and healthy controls. LMX1B plays a crucial role in the determination of dorsal-ventral limb patterning in the developing limb (3, 25). LMX1B mutant mice showed loss of dorsal features, such as the absence of the patella, and duplication of the ventral muscle and tendon pattern in the dorsal limbs at the level of the metatarsals (26). In the forelimbs, dorsal features (hair follicles) were missing and replaced by ventral features (foot pads), and the nails appeared to be missing. Taken together, nail deformities in patients with NPS are caused by the abnormal development of dorsal limb structures, rather than dysfunction of the EBM (Fig. 4).

Genetic analysis of NPS has so far determined that haploinsufficiency of LMX1B leads to the clinical manifestation of NPS (27). In this study, we evaluated 2 families with NPS. Direct sequencing analysis revealed that family 1 and family 2 were heterozygous for the splice-site mutations, c.140-1G>C (IVS1-1G>C) and c.326+1G>C (IVS2+1G>C) in LMX1B, respectively. To identify the mechanism of pathogenesis, we performed RT-PCR of cDNA extracted from the dorsal skins of their thumbs, which showed normal and shortened PCR products in both samples. Intriguingly, sequencing of the shortened PCR products revealed the same splice variant with deletion of exon 2, leading to a frameshift and a downstream premature termination codon (p.Asp49Serfs*17). Our data confirm that NPS is caused by haploinsufficiency of LMX1B as previously reported (27).

In conclusion, we describe here 2 families with NPS, both heterozygous for the distinct splice-site mutations in LMX1B, resulting in skipping of exon 2. Electron microscopy and immunohistochemistry were used to analyse the EBM zone: no significant differences in EBM were found between patients with NPS and the healthy control group. This study suggests that nail dysplasia in NPS patients is not caused by abnormalities of the EBM.

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