

Hereditary Leukonychia, or Porcelain Nails, Resulting from Mutations in *PLCD1*

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Hereditary leukonychia (porcelain nails or white nails) is a rare nail disorder with an unknown genetic basis. To identify variants in a gene underlying this phenotype, we identified four families of Pakistani origin showing features of hereditary leukonychia. All 20 nails of each affected individual were chalky and white in appearance, consistent with total leukonychia, with no other cutaneous, appendageal, or systemic findings. By using Affymetrix 10K chip, we established linkage to chromosome 3p21.3-p22 with a LOD score (Z) of 5.1. We identified pathogenic mutations in *PLCD1* in all four families, which encodes phosphoinositide-specific phospholipase C delta 1 subunit, a key enzyme in phosphoinositide metabolism. We then identified localization of *PLCD1* in the nail matrix. It was recently shown that *PLCD1* is a component of the human nail plate by proteomic analysis and is localized in the matrix of human nails. Furthermore, mutations detected in *PLCD1* resulted in reduced enzymatic activity in vitro. Our data show that mutations in *PLCD1* underlie hereditary leukonychia, revealing a gene involved in molecular control of nail growth.

The nail is a specialized skin appendage with a complex structure involving a fully keratinized nail plate produced by the germinative epithelium of the nail matrix. Significant advances have been made in the diagnosis and management of nail diseases, yet our knowledge of the underlying molecular controls of nail growth and development is relatively scant.¹ A white appearance of nails can result from whitening of the nail plate (true leukonychia), the nail bed (pseudoleukonychia), or neither (apparent leukonychia) and can be due to a variety of factors including infectious, metabolic, or systemic diseases, trauma, or drugs.² One of the rare causes of whitening of the nail plate is hereditary leukonychia (MIM 151600), a rare autosomal-dominant or -recessive nail disorder with an unknown genetic basis. In hereditary leukonychia, whitening of the nails may exist as an isolated feature or be associated with other cutaneous or systemic abnormalities, including keratosis palmoplantaris, pilar and sebaceous cysts, severe keratosis pilaris, pili torti, hypotrichosis with keratoderma, onychorrhexis, koilonychia, renal calculi, and hair dysplasia with acanthosis-nigricans-like lesions.²⁻¹⁴

We undertook this study to identify a gene involved in hereditary leukonychia, with the aim of defining a new genetic determinant of nail growth in humans. We identified four families of Pakistani origin showing features of hereditary leukonychia that were present since birth. Two of the families included consanguineous marriages or other features consistent with recessively inherited leukonychia (Figure 1C), whereas the other two families demonstrated dominant inheritance (Figure 1C). All 20 nails of each affected individual were chalky white, consistent with total leukonychia (Figure 1A), although some

displayed incomplete leukonychia with translucency and yellowish coloration in the distal parts of the nail plate. There was a negative family history for malignancy. No other skin, hair, dermatologic, or systemic findings were detected. Informed consent was obtained from all subjects and approval for this study was provided by the Institutional Review Board of Columbia University. The study was conducted in adherence to the Declaration of Helsinki Principles. Peripheral blood samples were collected from members of four Pakistani families as well as 130 unrelated healthy control individuals of Pakistani origin. Genomic DNA was isolated from these samples according to standard techniques.¹⁵ We performed a genome-wide scan on eight members of family A with the low-density Affymetrix 10K SNP array. Genespring GT (Agilent Software) was used for quality control measures and to perform a number of analyses. SNPs showing Mendelian inheritance errors were removed, and the remaining analyzed data set contained 9887 SNPs. Haplotypes, or clusters of SNPs that tend to be inherited together, were inferred from the data by Genespring GT. Using haplotypes minimizes the effect of linkage disequilibrium on multipoint linkage analysis, reducing type II error. Initial analysis included genome-wide autozygosity mapping to identify regions identical by descent that were shared among affected individuals. We performed parametric linkage analysis twice, once with SNP genotypes and once with haplotypes. All tests assumed a recessive mode of inheritance with 100% penetrance and a disease allele frequency of 0.001. We identified a region of excess homozygosity shared among affected individuals on chromosome 3p21.3-p22 (Figure 1B) with a LOD score (Z) of 5.1. Microsatellite markers spanning the region of autozygosity were then used to

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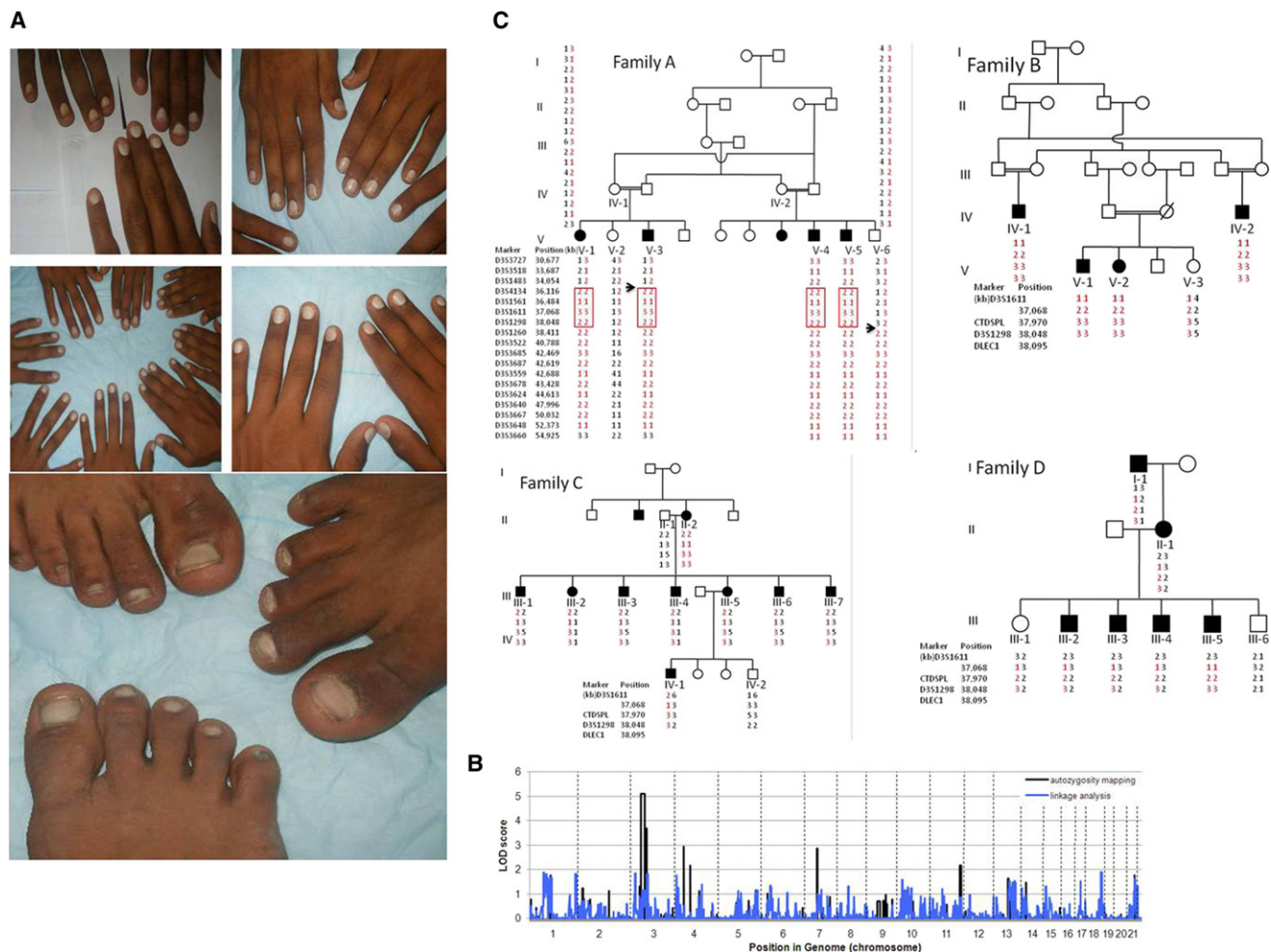


Figure 1. Clinical Features of Affected Individuals and Linkage to Chromosome 3 and Identification of Mutations in *PLCD1*
 (A) Complete nail plate leukonychia involving all the finger and toe nails in affected individuals from both recessive families.
 (B) Results of autozygosity mapping in family A. The maximum LOD score was obtained for a region on chromosome 3.
 (C) Haplotype analysis in families A, B, C, and D. The linked haplotype is indicated in red, and key recombination events are indicated by arrows.

genotype all families, revealing linkage to the same location in the other three families (Figure 1C). Key recombination events were detected between markers D3S1483 and D3S4134 in the affected individuals V-1 and V-3 of family A (Figure 1C) and between markers D3S1298 and D3S1260 in the unaffected individual V-6 (Figure 1C), which allowed the interval of linkage to be narrowed to 2.0 Mb flanked by markers D3S1483 and D3S1260.

The region of interest harbored 18 known genes, 15 pseudogenes, and 3 predicted transcripts (Figure 2A). After excluding genes associated with other inherited disorders and sequencing seven known genes in the region (*STAC* [MIM 602317], *ITGA9* [MIM 603963], *DCLK3* [MIM 613167], *SLC22A13* [MIM 604047], *GOLGA4* [MIM 602509], *SLC22A14* [MIM 604048], and *PLCD1* [MIM 602142] [NM_001130964.1]), we identified predicted mutations in *PLCD1* in all four families (Figures 2B and 2C). All exons of *PLCD1* were amplified by PCR with gene-specific primers (see Table S1 available online). The PCR products were directly sequenced in an ABI Prism

310 Automated Sequencer, with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). We screened for the *PLCD1* mutations in genomic DNA from 130 unrelated healthy control individuals (260 chromosomes) of Pakistani origin. Affected individuals of autosomal-recessive family A displayed a homozygous mutation c.1309C>T leading to a premature termination codon p.Arg437X (Figure 2B). The affected individuals in the second family with autosomal-recessive inheritance, family B, also showed a homozygous protein-truncating mutation consisting of a 10 bp deletion spanning the splice junction of intron 11 and exon 12 (c.1792-10delTGTAGTGGCC) resulting in a premature termination codon because of either a frameshift or missplicing (Figure 2C). By contrast, affected individuals of families C and D with autosomal-dominant inheritance displayed heterozygous missense mutations c.1720C>T (antisense strand) and c.625T>C in *PLCD1* leading to the amino acid substitutions p.Ala574Thr and p.Cys209Arg, respectively (Figures 2D and 2E). None of

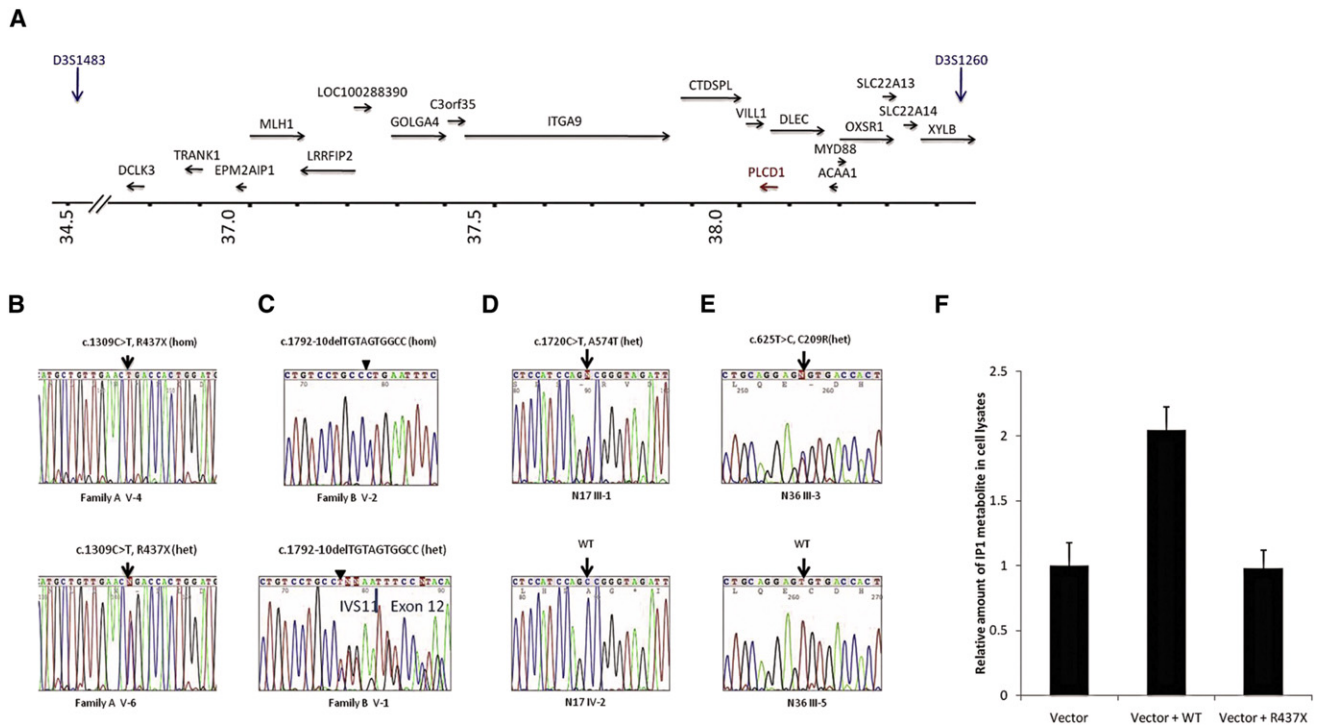


Figure 2. Identification of Mutations in *PLCD1* and Function of Mutant *PLCD1*

(A) Schematic representation of the candidate region harboring *PLCD1*. Arrows indicate the position and the direction of transcription of genes in the region. *FCHP*, *LOC100130503*, *ARPP21*, and *STAC* are located upstream of the region shown where the axis has been truncated.

(B and C) Mutation in *PLCD1* in families A and B.

(B) Family A; homozygous mutation c.1309C>T, p.Arg437X in an affected individual V-4 (top); heterozygous mutation in an unaffected carrier V-6 (bottom).

(C) Family B; homozygous mutation c.1792-10delTTGATGGGCC in affected member V-2 and heterozygous in carrier member V-1 of family B.

(D) Heterozygous c.1720C>T (antisense strand), p.Ala574Thr in family C.

(E) Heterozygous c.625T>C, p.Cys209Arg in family D.

(F) Function of p.Arg437X mutant *PLCD1* protein in vitro by detection of phospholipase C downstream metabolite IP1. The quantity of IP1 was measured by ELISA 48 hr after transfection of HEK293T cells with vector only (negative control), vector and wild-type *PLCD1* construct (positive control), and vector and mutant *PLCD1* construct. The test was repeated five times and difference was statistically significant ($p < 0.01$).

the mutations were found in 130 population-matched, unrelated, unaffected control individuals screened by restriction enzymes (data not shown) or direct sequencing (for p.Ala574Thr and p.Cys209Arg, data not shown). p.Ala574Thr and p.Cys209Arg are both highly conserved among several organisms (Figure S1A). The identification of protein-truncating mutations indicated that an absence or reduction of *PLCD1* activity might underlie the leukonychia phenotype in the affected individuals. To confirm this hypothesis, we assayed the function of the p.Arg437X mutant *PLCD1* in vitro. The wild-type *PLCD1*-cDNA was amplified by PCR from human foreskin total mRNA with the following primers: forward 5'-AAACTCG AGGCCACCATGCAGTGCCTGGGGATC-3' and reverse 5'-AAAGGTACCGCCTAGTCCTGGAGGGAGATC-3'. The PCR product was cloned into the mammalian expression vector pCXN2.1¹⁶ with XhoI and KpnI sites. The expression construct for the p.Arg437X mutant *PLCD1*-p.Arg437X-pCXN2.1 was generated with the QuikChange II XL Site-Directed Mutagenesis Kit (QIAGEN) with the

PLCD1-pCXN2.1 construct as a template. To measure *PLCD1* function, accumulation of its downstream metabolite, inositol monophosphate (IP1), was quantified with IP-One ELISA competitive immunoassay kit (Cisbio). We plated 1.5×10^5 HEK293 cells on 24-well plates and transfected with pCXN2.1 (vector only), the wild-type *PLCD1* containing construct (*PLCD1*-pCXN2.1), or the mutant (p.Arg437X) *PLCD1* containing construct (*PLCD1* p.Arg437X-pCXN2.1) with Fugene transfection reagent (Invitrogen). Forty-eight hours after transfection, cells were stimulated in the presence of LiCl causing the accumulation of IP1 upon receptor activation and cell lysates were collected. Competitive immunoassay with IP1-HRP and a IP1 monoclonal antibody was then carried out according to manufacturer's instructions. The optical density (OD) was read at 450 nm with BioRad microplate reader model 680.

We found a significant reduction ($p < 0.01$) in IP1, a downstream metabolite of inositol 1,4,5-trisphosphate (IP3) that accumulates in cells after activation of phospholipase

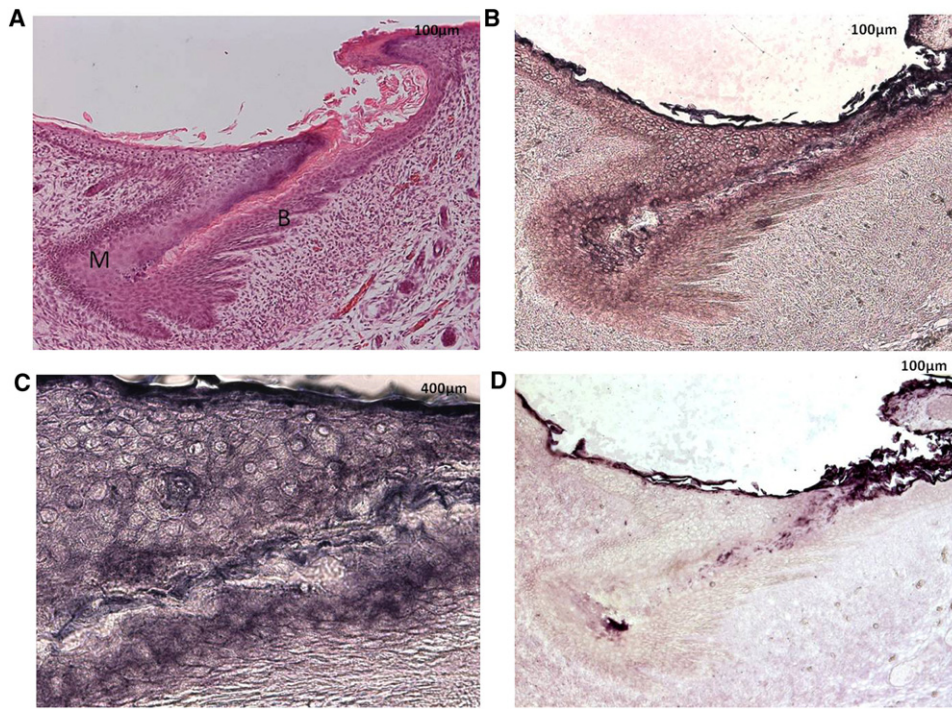


Figure 3. PLCD1 Localization in Human Embryo Nail

(A–C) Anatomy of human embryo nail by (A) hematoxylin and eosin staining (M, nail matrix; B, nail bed) and by (B and C) PLCD1 staining (purple indicates positive staining) revealing cytoplasmic and membranous localization of PLCD1. (D) Negative control.

C-coupled receptors, PLCD1 in 293 cells transfected with the mutant construct compared with the wild-type (Figure 2F).

To further implicate PLCD1 in the pathogenesis of hereditary leukonychia, we examined its localization in the nail. For PLCD1 immunohistochemistry, paraffin-embedded sections of human fetal (IRB-AAAB2447) nail were used. Antigen unmasking was carried out with Antigen Unmasking Solution (Vector Laboratories). After blocking with normal goat serum, samples were incubated with a rabbit polyclonal antibody raised against human PLCD1 (1:50 dilution; HPA020107; Sigma Prestige) at 4°C overnight in a humidified chamber. Primary antibody was omitted from the negative control. To visualize the staining, the Vectastain Elite rabbit ABC-AP kit (Vector Laboratories) and alkaline phosphatase (AP) staining with 5-Bromo-4-chloro-3-indolyl phosphate and 4-Nitro Blue Tetrazolium Chloride (Roche Applied Science) in AP staining buffer (100 mM Tris [pH 9.5], 50 mM MgCl₂, 10 mM NaCl, and 1% Tween 20 in H₂O) were used. By immunohistochemistry analysis, prominent localization was detected in the nail matrix and the nail bed (Figures 3A–3D). We then isolated total RNA from plucked human scalp hair, human scalp, human foreskin, and foreskin-derived keratinocytes and fibroblasts with the RNeasy Minikit (QIAGEN). 2 µg of total RNA was reverse transcribed with random primers and SuperScript III (Invitrogen). The cDNAs were then amplified by PCR with *PLCD1*-specific primers (forward 5'-CAGCGTCAGAAGCTACAGCA-3',

reverse 5'-TCTGTCTGGGAGTGGTCACA-3') and SYBR Green master mix. The PCR reactions were run in an Applied Biosystems Sequence Detection System 7700. Relative expression levels were calculated with the $\Delta\Delta Ct$ method (Applied Biosystems), with *GAPDH* as the internal control. The expression level of human scalp was set as the calibrator. Although *PLCD1* mRNA was expressed in human skin and hair follicle as evidenced by real-time quantitative RT-PCR analysis (Figure S1B), we found no evidence of hair or skin abnormalities in the affected individuals.

Although many hereditary disorders with isolated or combined nail defects have been described, the underlying gene defect in most of these diseases is yet unknown.¹ Some of the genes implicated in human nail diseases include *LMX1B* (MIM 602575), a transcription factor that regulates collagen IV expression in the nail-patella syndrome (NPS [MIM 161200]),¹⁷ *K6* (MIM 148041), *K16* (MIM 148067), and *K17* (MIM 148069) in pachyonychia congenita type 1 (PC1 [MIM 167200]) and pachyonychia congenita type 2 (PC2 [MIM 167210]), a group of rare autosomal-dominant ectodermal dysplasias,¹⁸ and *RSPO4* (MIM 610573), a Wnt signaling ligand we found to be mutated in congenital anonychia (MIM 206800).¹⁹ Previously, Norgett et al.²⁰ showed genetic linkage in a family with autosomal-dominant hereditary leukonychia to the type II cytokeratin locus on 12q13 and detected abundant intracellular vacuoles and a lesser compactness of keratins in the affected nails, postulating that the white appearance

Table 1. Summary of the Four Families and Their Mutations

Family	Pattern of Inheritance	Mutation	Consequence
A	autosomal recessive	c.1309C>T, p.Arg437X	suppresses IP1, a downstream metabolite of PLCD1
B	autosomal recessive	c.1792-10delTGTAGTGGCC	not tested
C	autosomal dominant	c.1720C>T (antisense strand), p.Ala574Thr	not tested
D	autosomal dominant	c.625T>C, p.Cys209Arg	not tested

of nails seemed to be due to an abnormal keratinization of cells originating from the proximal nail matrix.

The finding of *PLCD1* mutations in four families with autosomal-recessive and autosomal-dominant hereditary leukonychia (Table 1) broaden our knowledge of genetic control of nail growth in humans and emphasize the involvement of nonkeratin genes in these processes.

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in phosphoinositide turnover by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, diacylglycerol (DG) and IP3.²⁰ DG further mediates the activation of protein kinase C, and IP3 releases calcium from intracellular stores playing a role in a variety of physiological functions.²¹ PLCs are divided into six isotypes: four β , two γ , four δ , one ϵ , one ζ , and one η types. The catalytic domains of all PLCs share 40%–60% similarity. These isotypes are regulated by different mechanisms. For instance, PLC- β isoenzymes are activated by the *G α q/11* family of heterotrimeric G-proteins and $\beta\gamma$ subunits are released from the *G α i/o* family while PLC- γ isoenzymes are activated by tyrosine kinases through tyrosine phosphorylation. PLC- ϵ is activated by *G α 12* and ras through membrane translocation. These isotypes are expressed in various tissues where they are believed to play fundamental roles in triggering cellular responses.²² Recently, it was found that *PLCD1* acts as a tumor-suppressor gene in cell lines of gastric and breast cancer.^{23,24} None of our families in this study reported any history of malignancies.

Interestingly, *Plcd1*-deficient mice undergo progressive hair loss because of epidermal hyperplasia, occlusion of the hair canal, sebaceous gland hyperplasia, and formation of abnormal cysts, though a nail phenotype was not described.²⁵ Furthermore, *Plcd1*-knockout mice share striking phenotypic similarity to the *Foxn1*-deficient nude mice, whose skin defects are caused in part by insufficient expression of *Plcd1*.²⁶ Further studies are needed to examine the link between *Plcd1* and the nail dystrophy in nude mice. Nude mice also have nail dystrophy with shorter and abnormally shaped nails, severe parakeratosis of the dorsal nail plate because of dysplasia and impaired

cornification of the nail matrix, and lower nail plate sulfur concentrations.²⁷ Although *PLCD1* mRNA was expressed in human skin and hair follicle as shown by real-time quantitative RT-PCR analysis, we found no evidence of hair or skin abnormalities in the affected individuals, suggesting that other members of the phosphoinositide-specific phospholipase C family may compensate for the *PLCD1* defect in the skin and the hair follicle.

Most recently, it was shown through proteomic studies that *PLCD1* is abundant in human nails.²⁸ The mechanism through which it leads to the formation of leukonychia remains to be determined. The phenomenon that recessive and dominant mutations in the same gene result in a similar phenotype is very rare. Ataxia telangiectasia (AT [MIM 208900]) is an autosomal-recessive condition caused by mutations in *ATM* (MIM 607585). In 2008, Smirnov and Cheung investigated the effect of heterozygous and homozygous *ATM* mutations on gene and microRNA expression and found that some genes and/or pathways require only one wild copy of *ATM* to function normally whereas others require two copies. In summary, they demonstrated that mutations in *ATM* result in both recessive and dominant expression phenotypes of genes and microRNAs. Moreover, recessive and dominant mutations in keratins 5 and 14 have been reported in epidermolysis bullosa simplex (EBS).^{29,30} This is one possibility that can explain the occurrence of white nails in the setting of recessive or dominant mutations. A second possibility would be that the missense mutations exert a dominant-negative effect on the wild-type allele with complete loss of function.³¹

In conclusion, our findings indicate that mutations in *PLCD1* underlie hereditary leukonychia and, more broadly, reveal a gene involved in controlling nail growth in humans.

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at <http://www.cell.com/AJHG/>.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Agilent, <http://www.chem.agilent.com/en-US/Products/software/lifesciencesinformatics/Pages/gp63308.aspx>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

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