MUTATION IN PHOSPHOLIPASE C, δ1 (PLCD1) GENE UNDERLIES HEREDITARY LEUKONYCHIA IN A PASHTUN FAMILY AND REVIEW OF THE LITERATURE

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ABSTRACT
Human hereditary leukonychia is a rare nail disorder characterized by nail plates whitening on all finger and toe nails. Inheritance pattern is both autosomal dominant and recessive. To date, the only gene, phospholipase C, δ1 (PLCD1), on chromosome 3p22.2 has been reported to be involved in hereditary leukonychia. In the present study, a family of Pakhtun ethnicity, carrying leukonychia phenotype was investigated. The family inherited the phenotype in an autosomal dominant fashion. Affected individuals exhibited characteristic features of hereditary leukonychia with involvement of nails on both the hands and feet. Sequence analysis of DNA detected a p.Cys209Arg mutation, reported for the first time in a Pakistani Pashtun family.

Keywords: Leukonychia; Mutation; Pashtun family; Phospholipase C, δ1 (PLCD1) gene.

INTRODUCTION
Initiation of nail differentiation and growth is started round about the 9th week of gestation and its structure becomes completed within the 5th month. In the beginning, nails appear as a spot close to the 10th week of gestation, which is similar to hair placode and increases in length from distal to proximal end, the latter is converted into nail fold due to the differentiation of nail stem cells that are present in the region of the proximal end. Nail is produced by the matrix and grows over the nail bed [1].

As a result of matrix epithelial cell differentiation, the mature nail plate grows continuously through life and consists of a number of hard and soft keratin molecules embedded in an amorphous matrix. Abnormal keratinization of these matrix cells is considered to be responsible for the white appearance of nails in hereditary leukonychia. Most inherited nail disorders manifest either with nail hypoplasia or nail hypertrophy [2].

Based on the distribution of the white tone, leukonychia is classified into three different types. This includes true leukonychia, with the involvement of the nail plate originating in the matrix. In case of apparent leukonychia, the nail matrix is normal, however, involving subungual tissue causing alteration in the color of the overlying nail plate. The third type is pseudo-leukonychia, when the matrix is not responsible for the nail plate alteration. The nail plate is diseased because of external factors such as fungal infection of the nail. The true leukonychia is further separated into total and subtotal or partial, the latter occurring as leukonychia punctata, leukonychia striata, and leukonychia distal [3].

Hereditary leukonychia, characterized by whitening of the nails was mapped to chromosome 3p21.3-p22 with pathogenic mutations on the phospholipase C, δ1 (PLCD1) gene [4-6]. In the study presented here, we investigated a Pashto-speaking family from Lukki Marwat district of Khyber Pakhtunkhwa Province, the western part of Pakistan, segregating hereditary leukonychia in an autosomal dominant manner. Based on phenotypes, direct sequence analysis of the PLCD1 gene revealed a missense mutation.

MATERIALS AND METHODS

Human Subjects and DNA Samples. In order to investigate at the molecular level, a three-generation fami-
ily was collected from a remote region of Pakistan. Prior to commencement of the clinical and molecular investigations, written informed consent signed by the legal guardians, the parents, on behalf of the affected children, and they agreed to the publication of the study outcomes. Ethical approval of the study was obtained from the Institutional Review Board (IRB), Kohat University of Science and Technology (KUST), Khyber Pakhtunkhwa, Pakistan.

In order to identify the causative gene defect, peripheral blood samples were collected from four affected (II-2, III-1, III-2, III-3) and two unaffected individuals (II-1, III-4) in EDTA-containing vacutainer sets (Becton Dickin-son & Company, Franklin Lakes, NJ, USA) (Figure 1; Figure 1A). Genomic DNA was extracted from the blood by using Nucleospin® Blood kit (Macherey-Nagel, Düren, Germany). Nanodrop-1000 spectrophotometer (Thermal Scientific, Wilmington, NC, USA) was used for DNA quantification, measuring optical density at 260 nm and diluted to 40.0-50.0 ng/µL-1 for amplification by polymerase chain reaction (PCR).

**Candidate Gene (PLCD1) Screening.** Entire coding regions and splice junction sites of the gene were amplified by PCR and screened by DNA sequencing for potential sequence variants. The primer3 program (http://primer3.sourceforge.net/) was used to design intronic primer pairs for individual exons amplification, and basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/blast) was used to check for specificity. To obtain a DNA sequence for the gene, UCSC Human Genome browser (http://www.genome.ucsc.edu/cgi-bin/hgGateway) was used. Purification of the PCR-amplified DNA was performed with commercially available kits (Marligen Biosciences, Ijamsville, MD, USA). The amplification conditions were 5 min. at 95 °C., followed by 30 cycles of 12 seconds at 95 °C, 5 seconds at 50 °C, and 4 min. at 60 °C, with a final extension at 60 °C for 20 min. The amplified PCR products were sequenced using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Bioedit sequence alignment tool (Bioedit editor version 6.0.7) was used to align the sequence of each amplicon.

**Protein Structure Prediction.** The three-dimensional (3D) model of normal and mutant PLCD1 protein was predicted using the phosphoinositide-specific phospholipase c-delta1 structure (PDB ID 1DJZ) as a template. The structure for Cys209Arg mutant was developed by changing the selected residue into the desired residue and follow refinement of the structure by subjecting it to similar energy minimization protocol that was used for the wild structure. Comparative modeling was performed using molecular operating environment (MOE; https://www.chemcomp.com/MOE-Molecular_Operating_Environ-

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**RESULTS**

**Clinical Findings and Mutational Analysis.** All affected individuals of the family, studied here, showed typical features of hereditary leukonychia. These include chalky white, consistent with total leukonychia on their hands and feet. Whiteness of the nails involved the entire nail, including the lunula, and was present since birth. One affected individual (III-2) displayed incomplete leukonychia with yellowish coloration in the distal parts of the nail plate in the middle toe nail. All 20 nails had normal growth rates (Figure 1B). Other abnormalities of skin, hair, teeth and sweating were not observed in any affected member of the family.

Based on phenotypes, the already reported PLCD1 gene involved in the particular disorder on chromosome 3p21.3-p22, was planned for sequencing before embarking into the whole exome sequencing. Subsequently, all fifteen exons and splice sites of the PLCD1 gene were
sequenced in all available affected and unaffected members. Affected individuals of the family with autosomal dominant inheritance displayed heterozygous missense mutation involving a T to C transition at nucleotide position 625 in the *PLCD1* gene, resulting in the substitution of cysteine to arginine amino acid at position 209 (p.Cys209Arg) (Figure 2A). The variant is present in the genomeAD data-base in overall frequency of 0.00006095 in the heterozygous state.

A homology modeling techniques was used to identify the structural role of the mutated positions, we analyzed their intra-molecular interactions and compared wild-type with mutant model (Figure 2B-2D). We were able to identify intra-molecular changes only for the amino acid substitution at residue Cys209. In fact, Cys209 was not involved in any interaction with nearby residues, while in the mutant model, Arg209 forms hydrogen bonds with the nearby Ile145, which, due to the difference in bonding, may provide a local difference in the helix structure as seen from the figure (Figure 2C and 2D).

**DISCUSSION**

The present study describes a family with characteristic features of leukonychia. Affected individuals show typical chalky whitening of nail both in hands and toes. One of the affected members (III-2) also showed yellow pigmentation at the toe nails, as observed earlier in a family reported by Mir et al. [5].

This is the first Pashto-speaking family carrying a mutation at position p.Cys209Arg having leukonychia with autosomal dominant inheritance mode. In our present and previous report [5], we have studied several patients with mutations on the *PLCD1* gene, causing a leukonychia phenotype. However, we could not find any difference in the severity of the nails whitening in patients carrying different mutations, and no clear genotype-phenotype correlation emerged.

To date, only five distinct mutations have been identified in the *PLCD1* gene causing leukonychia. Of these, three mutations underlie the autosomal recessive form of leukonychia, while the other two mutations (including the mutation reported in this family) are responsible for the autosomal dominant form of the disease (Table 1). It appears that protein truncation mutations cause the recessive forms, while the autosomal dominant forms are caused by missense mutations. Possibly the missense mutation could exert a dominant negative effect on the wild-type allele with complete loss of function.

Analysis of the protein sequence by protein prediction tool PolyPhen2 [http://genetics.bwh.harvard.edu/]
pH2/]

The PLCD1 gene is composed of 15 exons. It spans a 22.17 kb region and encodes two isoforms containing 777 and 756 amino acids, respectively. It is a member of a large superfamily of phosphoinositide-specific phospholipase C (PLC), which is involved in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce second messengers including diacylglycerol (DG) and inositol triphosphate (IP3). As a result of PLCD1 gene disruption, a significant reduction of inositol monophosphate IP1 occurs, which is a downstream metabolite of IP3. PLC-δ1 is highly expressed in nail matrix, hair follicles, hair matrix and the nail bed [4,8].

It has been suggested that PLC-δ1 functions downstream of the FOXN1 gene transcription factor that regulates hard keratin gene expression essential for nail differentiation [9]. Interestingly, loss of function mutations in the FOXN1 gene results in defects of onychomelial differentiation and severe onychodystrophy in both mice and humans [10]. Therefore, loss of PLC-δ1 function may result in abnormal keratinization of nail plate due to aberrant expression of hard keratins causing leukonychia phenotype.

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